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GLYCOPROTEIN BIOSYNTHESIS: SOME ASPECTS OF REGULATION

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GLYCOPROTEIN BIOSYNTHESIS: SOME ASPECTS OF REGULATION

CHAPTER I

INTRODUCTION

Plasma Glycoproteins in Health and Disease

With the exception of albumin, almost all plasma proteins are glycoproteins and their concentrations, within limits, are fairly constant in healthy adult individuals. Seasonal and diurnal variations do occur, however (1). Glycoprotein levels are slightly higher in males as compared to females and there is a gradual increase in their concentrations in both sexes with advancing age (2). The protein-bound carbohydrates of fetal blood differ qualitatively and quantitatively from those of normal adults (3) and there is a marked increase in serum protein-bound hexose and hexosamine during pregnancy (4). These normal, physiological variations are of interest but most attention has been focused on the striking changes produced in the levels of a number of plasma glycoproteins by various pathological processes. Some of these are briefly discussed below.

Rheumatic Disorders

Increases in serum glycoproteins are associated with a number of inflammatory diseases, notably rheumatic fever and rheumatoid arthritis

(5). A positive correlation between the changes in serum levels of glycoproteins and the exacerbation of rheumatic diseases has been found in a number of cases (6), and Robinson and Roseman (7) have stated that all rheumatic diseases exhibit such changes in the acute stage. Cortisone therapy has been found to be effective in normalizing the elevated levels of protein-bound hexose in such cases (8). The increase in serum glycoprotein concentration is reflected mainly in the α_2-globulin and seromucoid fractions (9). Yachi et al. (10) identified some specific components of these fractions as orosomucoid, α_1-antitrypsin, and haptoglobin. The increased concentrations of these proteins were considerably reduced by treatment with anti-inflammatory agents like oxyphenbutazone and betamethasone.

Diabetes

In spite of a few contradicting reports (11, 12), it appears that uncomplicated diabetes mellitus is not associated with significant changes in serum levels of glycoproteins (13, 14). Protein-bound hexose and hexosamine, however, are increased significantly in diabetes mellitus complicated with degenerative vascular diseases (15). Serum glycoproteins have been found to increase by 60% in diabetic angiopathy (16).

Myocardial Infarction

Myocardial infarction leads to an increase in serum levels of α_1- and α_2-globulins including seromucoid and α_2-glycoprotein, with a maximum being reached on the sixth and seventh days (17, 18). The α_1-globulin level is more closely related to clinical severity than the α_2-globulin level. The serum level of albumin is simultaneously decreased.

Cancer

Plasma levels of some glycoproteins are significantly elevated in a number of neoplastic diseases (19-21). The C-reactive protein, which is usually not detectable in human plasma, is present in 60% of the patients with clinical cancer (22, 23). Lung cancer has been shown to be associated with hypoalbuminemia, an increase in α_1- and α_2-globulins and a lesser increase in β-globulins (24). The concentrations of hexose, hexosamine and sialic acid are considerably elevated in the plasma of children suffering from acute leukemia and their levels tend to decrease during clinical remissions of the disease (25). Perchlorate-soluble serum proteins (seromucoid) show a marked increase in the blood of people afflicted with sarcomas and carcinomas (26, 27).

A fetal glycoprotein closely related to fetuin has been reported to appear in the plasma of patients afflicted with primary liver hepatoma (28). It has been hypothesized that the synthesis of this protein, which is normally terminated at the time of birth, is resumed during oncogenesis by cells which have returned to a more primitive state.

Marked increases in the levels of serum protein-bound hexose, hexosamine, fucose, and sialic acid have also been found in Hodgkin's disease (29, 30), but attempts to correlate their indices with the clinical state have not been fruitful. The appearance of C-reactive protein in significant amounts in the sera of patients with Hodgkin's disease is a typical observation (31).

Infections

Increases in plasma glycoproteins have been found in clinical and experimental tuberculosis (32), pneumonia (32, 33), and a number of

other infectious diseases (34) and may indicate a general response to tissue damage and stress.

Trauma

Alterations in the level of plasma glycoproteins are not unique to the diseased state. Similar effects can be produced by tissue injury and experimental inflammation (stress) in a wide variety of forms. Weimer et al. (35) have detected pronounced elevations in fibrinogen, seromucoid, and in α_1-, and α_2-globulins after adjuvant-induced arthritis. Accidental and surgical trauma, bone fractures, hemorrhage, burns, freezing, γ-irradiation, intense physical exercise and a number of environmental factors cause marked increases in α-globulins, reaching a maximum between 24 to 48 hours after the stimulus (36-38). Injections of sterile Celite, talcum, turpentine, croton oil, and subcutaneous sponge implantation have been widely used to produce the response. The stress-responsive glycoproteins, referred to by many authors as "acute phase reactants," primarily include haptoglobin, orosomucoid, the α_1-acid glycoprotein of Darcy and that of Shultze, ceruloplasmin, α_1-antitrypsin, C-reactive protein, fibrinogen, and α_2-acute phase (α_2-AP) globulin (39-48).

The increases in serum proteins seen after stress do not result from a decrease in their catabolic rates; in fact, their catabolism, if affected at all, is slightly increased (36, 38, 49). Furthermore, the stress-induced increases can be abolished by injections of actinomycin D, ethionine, or puromycin, indicating that the elevated levels of these glycoproteins result from an increased rate of biosynthesis (50, 51).

Partial Hepatectomy

Since the liver has been established as the major site of

plasma protein synthesis (52), partial hepatectomy, in addition to injury, can also be used as a model for studying the regulation of glycoprotein metabolism. Although the circulating levels of most plasma proteins do not increase, but actually decrease after partial hepatectomy (52), the synthetic rates of most, if not all of them, are markedly increased. Chandler and Snider (53) and Majumdar et al. (54) have reported significant increases in the relative synthetic rates of albumin, fibrinogen, and the seromucoid fraction after partial hepatectomy. Mutschler and Gordon (55), and Infante et al. (56) have found similar increases in plasma protein synthesis in the isolated, perfused, regenerating rat liver. Thus, the decrease in the level of many plasma proteins observed after partial hepatectomy does not result from a diminution in their rates of synthesis but from an increase in their rates of degradation and from a reduced organ mass (57).

Significance of Plasma Glycoprotein Changes

In the light of rather scanty information available as to their true biological role, explanations regarding the physiological significance of an increase in the levels of certain plasma glycoproteins in response to stress can only be based on liberal conjecture. An increase in the level of α_1-antitrypsin may represent a need to inhibit proteolytic enzymes released after injury (58). Increases in haptoglobin may reflect a greater need for binding hemoglobin liberated as a result of hemolysis (36). Similarly, greater amounts of ceruloplasmin, transferrin and α_2-glycoprotein may be required to entrap copper, iron, and zinc, respectively, which are needed as cofactors in many enzymatic reactions and may also be toxic in high concentrations in the free form. Increased

quantities of α_2-macroglobulin may serve to bind various toxic substances set free during injury (59). Increased amounts of fibrinogen, of course, would accelerate blood clotting (36). Some glycoproteins are required for binding and carrying hormones. Reappearance of specific fetal and neonatal plasma proteins might play a part in the regeneration and repair of damaged tissue (60, 61). Some unusual proteins like C-reactive protein which appear in Hodgkin's disease and in some cancer subjects might have a role to play in the regulation of cell division (61).

Endocrine Regulation of Plasma Protein Metabolism

Hormonal regulation of plasma glycoprotein synthesis is one of the most extensively studied, yet most poorly understood, areas of glycoprotein metabolism. Most hormones appear to have a non-specific effect on glycoprotein synthesis and none of them seem to be absolutely necessary for inducing the stress-specific response. Some examples of endocrine involvement in plasma protein metabolism are discussed below.

Growth Hormone

Hypophysectomy in rats causes a reduction in the albumin and α_1-globulin fractions of serum proteins which is overcompensated by growth hormone (GH) administration (62, 63). Bovine GH can cause a significant increase in fibrinogen synthesis in rats within a few hours after administration (64).

The stimulatory effect of GH on plasma protein synthesis has also been demonstrated in a number of *in vitro* studies. Jefferson and Korner (65) were able to show that GH stimulates amino acid incorporation into proteins in the isolated, perfused rat liver. This stimulation

occurred, however, only when the concentration of the amino acids in the perfusate was raised several-fold over the normal. Similar results were obtained from experiments with liver slices (66).

Liver slices from hypophysectomized rats show a marked reduction in their ability to incorporate radioactive precursors into ribonucleic acid (RNA) and protein (67). The amount of polysomes is drastically reduced and their amino acid incorporating ability is badly impaired following hypophysectomy (68, 69). The degree of stimulation of phenylalanine incorporation by polyuridylic acid into disaggregated polysomes obtained from hypophysectomized animals is less than that into comparable preparations from the intact animals (70, 71). All of these parameters return to normal when hypophysectomized animals are treated with GH.

Barden and Korner (72) reconstituted rat liver ribosomes by hybridizing 40 and 60 S subunits isolated from normal and hypophysectomized animals and discovered that hypophysectomy specifically affected the 40 S subunit, since ribosomes containing this subunit from hypophysectomized rats showed a lower activity in amino acid incorporating systems. This work was contradicted, however, by Foster and Sells (73) who found that 80 S ribosomes prepared by reassociation, in all possible combinations, of 40 and 60 S subunits from normal and hypophysectomized animals were equally active in vitro.

The experimental evidence accumulated so far, indicates that GH stimulates protein synthesis in general. Even though plasma GH levels are elevated after surgery (74), GH probably does not exert a specific effect on the synthesis of individual serum proteins since the normal

response to stress is seen after hypophysectomy (75) and GH has little or no effect on plasma protein synthesis by the perfused liver (76). Tata (77) has, however, presented an idea that could explain selective increases in the synthesis of certain plasma proteins by GH. He has suggested that regulation of protein synthesis by growth and developmental hormones may be largely mediated through newly formed ribosomes. A topographical segregation and redistribution of polyribosomes by attachment to cytoplasmic membranes, which are generated in parallel with the additional ribosomes, could in this way facilitate the synthesis of specific proteins. The idea of differential synthesis of each plasma protein by a separate population of ribosomes seems to be attractive but needs more experimental support.

Insulin.

Insulin seems to exert a general effect on protein synthesis resembling that of GH. Liver microsomes from alloxan diabetic rats incorporate less amino acids into proteins than do microsomes from normal rats and insulin replacement restores full activity (78, 79). Liver perfusion studies (76) support earlier <u>in vivo</u> findings that insulin has a general stimulatory action on protein synthesis and is not in itself responsible for producing the stress-specific response. This effect on general protein synthesis may be observed to influence plasma protein synthesis under certain conditions. For example, Balegno and Neuhaus (80) have recently reported that the early stimulation of serum albumin synthesis following injury is dependent on insulin because this response to stress is suppressed in alloxan-diabetic rats and is restored by insulin therapy.

Adrenocorticotropic Hormone (ACTH) and Adrenocorticosteroids

One of the most striking aspects of the endocrine response to stress is the increased activity of the adrenal cortex. Most types of injuries in man and animals are accompanied by increased plasma levels of corticosteroids within 5 to 6 hours after injury (38, 75). This increase in plasma cortisol in man has been found to parallel increases in plasma levels of ACTH (36, 75, 81). Corticosteroids and ACTH produce an increase in serum hexosamine, and in dogs, cortisone increases the level of α_1-globulin (36). Injections of ACTH lead to a marked increase in plasma levels of fibrinogen in rats and rabbits (82).

Weimer (83) reported that adrenalectomy caused a pronounced reduction in the response of α_2-AP globulin and of seromucoid to tissue injury in rats. Moderate doses (0.6mg/100g) of cortisol fully restored the normal response to injury, but higher doses (>0.6mg/100g) were found to be inhibitory. John and Miller (76) were able to produce marked increases in fibrinogen, haptoglobin, α_1-acid glycoprotein and α_2-AP globulin synthesis in isolated, perfused rat liver by infusing cortisol, expecially when supplemented with insulin, GH and a complete amino acid mixture. These increases were critically dependent upon the presence of cortisol.

Leon (84) observed a 118% increase in the amino acid incorporating ability of liver microsomes isolated from rats 30 minutes after a single injection of corticosterone. Similarly, Angelov and Richter (85) showed a 200% increase in the uptake of tyrosine^{14}-C into liver microsomal proteins within 4 hours after hydrocortisone injections (1mg/g).

Intraperitoneal injections of hydrocortisone-21-phosphate

(1.4mg/100g) to rats have been claimed to increase the synthesis of DNA-like RNA by 32% and total nuclear RNA by 11% (86).

The work cited above supports the idea that the stress-induced increase in plasma glycoprotein synthesis may be mediated by adrenocortical hormones. This concept, though appealing, is not all that clear. There are many reports in the literature that support the opposite point of view. For example, Kataja and Staehlin (87) observed an increase in leucine-^{14}C incorporation into rat liver ribosomes following adrenalectomy. Ribonucleoprotein-particles from livers of adrenalectomized rats were found to incorporate twice as much leucine-^{14}C into protein as did those from normal animals (88); treatment with corticosterone abolished this effect. Koike et al. (89) have observed an early stimulation (30-60 min.) of protein synthesis followed by a marked depression (4-5 hours) in livers from adrenalectomized mice after cortisone administration. Hydrocortisone has been reported to depress RNA and protein synthesis in plasmacytoma Tu X-5563 cells (90).

Thus it is clear that the effects of corticosteroids on protein metabolism are exceedingly complex. The marked discrepancies in results obtained by various investigators after ACTH and corticoid treatment are probably due to differences in dosage, timing, species and other factors. In addition, it is also apparent that the corticosteroids exert different effects on different proteins; i.e., stimulate the synthesis of some and depress that of others.

As far as the acute-phase response to stress is concerned, it appears that, although the corticosteroids play an important role in inducing a proper response, they are not the sole factors involved. It

has been reported, for example, that adrenalectomy does not abolish the increase in the seromucoid fraction or the increase in amino acid incorporation into microsomes normally observed following stress. It does, however, cause a marked shift in the pattern of response; i.e., the response is delayed (91). Replacement therapy with glucocorticoids restores a normal pattern of response, but only if initiated during the early phase of the response. Excessive doses (3.75 mg/100g) of cortisone suppress the seromucoid response in adrenalectomized rats and stimulate the synthesis of serum albumin.

Experiments like these, and others, have led various investigators (36, 75, 91) to propose that corticosteroids have only a permissive role to play in plasma protein synthesis—— their presence is necessary for the metabolic response to occur but they are not directly involved in producing the specific changes.

Very little is known about the mechanism of action of these hormones. Beato et al. (92) studied the subcellular distribution of radioactivity in rat liver after intraperitoneal injections of tritiated cortisol and found that the bulk of radioactivity was in the cytoplasmic fraction; only 0.2-0.4% was recovered bound to protein in purified nuclei. About 10% of the cytoplasmic radioactivity existed as cortisol associated with protein. The authors suggested that cortisol exerted its effect on RNA synthesis by binding to a specific protein having a regulatory function. Louisot and coworkers (93, 94) have reported that injections of 3', 5'-cyclic-AMP can increase the steroid binding activity of transcortin at the subcellular level in rat liver cells with a simultaneous increase in glucosamine-^{3}H incorporation into glycoproteins.

A general increase in liver tRNA synthesis following cortisone injections has been seen by Agarwal et al. (95, 96).

Various models for the transcriptional and translational control of protein synthesis by hormones have been presented by Tata (97), Kenney (98), O'Malley (99), Tomkins (100), Britten and Davidson (101), and Budavari (102). Although each of these has some attractive features for a specific system or a specific hormone, it is clear that none adequately accounts for all the phenomena observed.

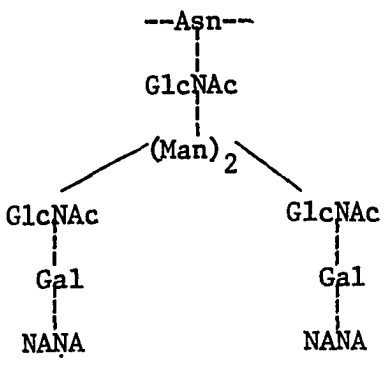
Initiation and Relay of the Stress Response

The process by which the "stress signal" from a remote site reaches the liver is another equally puzzling and intriguing aspect of the stress-induced increase in hepatic glycoprotein synthesis. Most conditions, discussed in the beginning of this chapter, that cause marked alterations in the levels of plasma proteins do not directly affect the liver. Thus there must be a specific mechanism for relaying the signal to this organ. Cuthbertson (38) has presented evidence suggesting that such a response could be mediated by an indirect stimulation of the CNS by afferent impulses originating at the site of injury. A number of experiments involving transections of various nerves or the spinal cord seem to support this idea. Injury to an animal whose pituitary is isolated from hypothalamic control by dividing the stalk, however, is capable of causing a brisk adrenocortical response (75). Consequently, a number of investigators (75, 103-106) have suggested that the response is produced by some humoral factor(s) or local hormone such as histamine, 5-hydroxytryptamine, bradykinin, or acetylcholine released from the damaged tissue, which could directly stimulate the adrenal cortex.

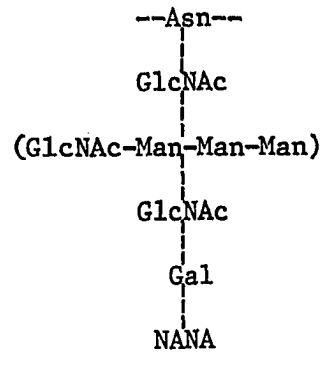
Structure of Glycoproteins

A knowledge of the structure of glycoproteins is helpful for understanding the mechanisms of their biosynthesis and regulation. Glycoproteins are distinguished from other proteins by the presence of one or more carbohydrate moieties covalently linked to the polypeptide chains. The sugars most commonly found in plasma glycoproteins are: N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, D-mannose, D-galactose, L-fucose, and sialic acids. L-Fucose and sialic acids have invariably been found to occupy a terminal position at the non-reducing end of the carbohydrate chains. The innermost sugar residue that links the heterosaccharide chain to the polypeptide backbone is either N-acetylglucosamine bonded to the amide nitrogen of asparagine (107), or N-acetylgalactosamine linked to the hydroxyl group of serine or threonine (108). Both linkages are found in plasma glycoproteins, but the predominant linkage is that to asparagine. The core of the heterosaccharide chains is usually made up of mannose and N-acetylglucosamine residues.

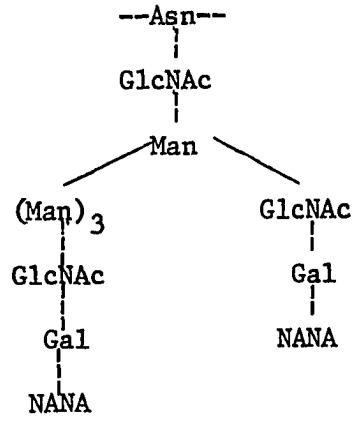
Although the carbohydrate composition of many glycoproteins is known in terms of the percentage and number of each sugar residue per mole, the exact sequence of sugars in the heterosaccharide chains of each glycoprotein has been determined in only a relatively few cases. Sugar sequences proposed for some glycopeptides of the type found in plasma glycoproteins are shown as follows:



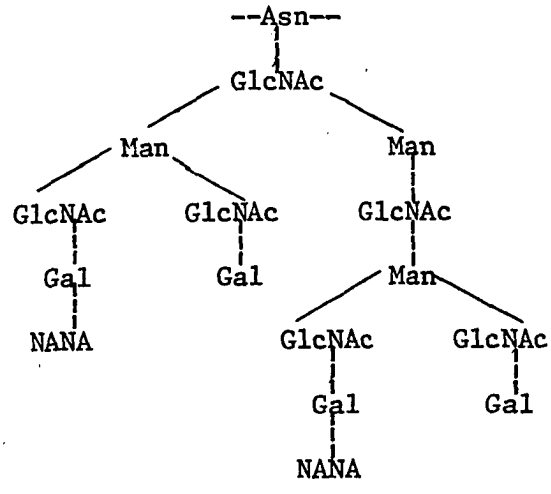
A glycopeptide from human erythrocyte membrane (109)



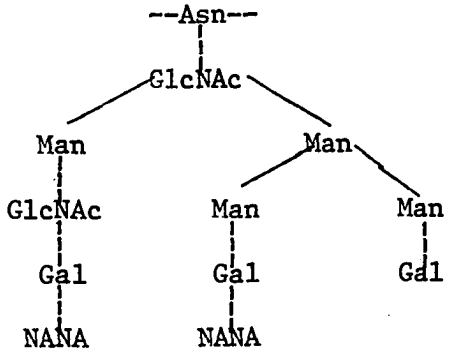
A glycopeptide from fetuin (110)



A glycopeptide from transferrin (111)



A glycopeptide from α_1-acid glycoprotein (112)



A glycopeptide from fibrinogen (113)

In spite of considerable variations found in the number and sequence of sugar residues in the carbohydrate moieties of various plasma glycoproteins, a general structural pattern can be formulated. N-Acetylglucosamine, the innermost residue linking heterosaccharide chains to the polypeptides, is usually followed by a mannose residue(s). Branching of the chain may take place at this point or at subsequent

residues. Mannose is again followed by N-acetylglucosamine and this sequence may be repeated again. Galactose is subsequently attached to an N-acetylglucosamine residue, usually at the terminal or penultimate position. L-Fucose and sialic acid, when present, always occupy the terminal position at the non-reducing end of the chain. Thus a general pattern of the sugar sequence in the oligosaccharide chains of most plasma glycoproteins can be depicted as:

Asn-(GlcNAc)-(Man)-(GlcNAc)-(Gal)-(sialic acid or L-fucose).

Although the structure of each carbohydrate moiety of a given glycoprotein is relatively specific, variations in carbohydrate structure have been found even between molecules having the same amino acid sequences in the polypeptide portion. Thus the composition of a single oligosaccharide prosthetic group of ovalbumin is not identical in all ovalbumin molecules; the ratio of two sugar constituents, mannose and N-acetylglucosamine, have been reported to vary from 2.6 to 1.1 (114). In bovine pancreatic ribonuclease B, mannose and N-acetylglucosamine were found in ratios varying from 2.5 to 3.0 (115). This type of "peripheral microheterogeneity," caused by variations in the peripheral residues, has been reported for α_1-acid glycoprotein (116), fetuin (117), α_2-macroglobulin (118), transferrin (119) and most other glycoproteins studies. "Central heterogeneity" is found in IgG (120), where one heterosaccharide is linked by N-acetylglucosamine to an asparagine residue and another by N-acetylgalactosamine to a threonine residue. Myeloma globulin A has three distinct heterosaccharides, two linked to asparagine and one to serine (121). Similarly, thyroglobulin has been found to contain two different types of heterosaccharide units (122).

The physiological or pathological significance of "peripheral" or "central" microheterogeneity is not known as yet but certainly deserves further consideration and careful study.

Biological Role of the Carbohydrate Moiety of Glycoproteins

A simple protein owes its specificity and structural complexity to the kind, number and sequence of amino acids that make up its polypeptide chain(s). A glycoprotein supersedes simple proteins in these properties by virtue of its additional carbohydrate side chains which provide means for further specificity and diversification. The presence of negatively charged sialic acid and the relatively more lipophilic L-fucose residues provides glycoproteins with additional opportunities for interactions with other macromolecules, especially in membranes; consequently, glycoproteins should be capable of carrying on more specific and versatile functions than simple proteins.

Glycoproteins indeed participate in a wide variety of reactions such as blood-clotting, antigen-antibody interactions, freezing-point depression, regulation of organ growth, and metal and hormonal transport. They also serve as virus receptors, act as lubricants, filtration barriers and supportive elements and provide protection from proteolytic enzymes (123-125). The antigenic activities of A, B, H and Lewis blood group substances are certainly manifestations of the structure of their heterosaccharide chains (126). Pazur et al. (127) have proposed that the carbohydrate moieties stabilize the three-dimensional structure of some glycoenzymes and in turn influence their catalytic activities.

Since most glycoproteins are exported from the cells in which they are synthesized, Eylar (128) has suggested that the carbohydrate

portion of glycoproteins may take part in the process of transport by interacting with cell membranes and thus facilitating secretion. This interaction may also help in the reverse process when glycoproteins enter cells. Kukral et al. (129) observed that the appearance of radioactivity in the liver following intravenous injections of ^{125}I-labeled haptoglobin into dogs was dependent on the content of sialic acid in the haptoglobin molecule. Unmodified haptoglobin penetrated more freely into extravascular spaces and was excreted more easily from the body than asialohaptoglobin. These observations seem to be in agreement with Eylar's hypothesis. Not all secreted proteins are glycoproteins, however. Albumin and insulin are good examples of proteins which lack carbohydrate side chains and are secreted in large quantities. Therefore, Eylar's hypothesis does not seem to be applicable to all exportable proteins.

Another, better-documented role for the carbohydrate moiety appears to be in the protection of the glycoprotein molecule from digestion by proteolytic enzymes. Morell and coworkers (130) have found that desialyzed plasma proteins including orosomucoid, fetuin, ceruloplasmin, haptoglobin, α_2-macroglobulin, and thyroglobulin are more promptly removed from circulation and degraded in the liver than are their normal counterparts. Similarly, ammonium sulfate-fractionated fetuin is resistant to digestion by trypsin and α-chymotrypsin until sialic acid is first removed (131).

One approach for elucidating the exact role of the carbohydrate portion of a glycoprotein molecule involves the stepwise removal of its sugar residues to see the impact of this treatment on the biological activity of the protein. Removal of sialic acid results in complete

loss of biological activity of erythropoietin, human chorionic gonadotropin and follicle stimulating hormone (126). Its presence is also necessary for antigenic activity of M and N blood group substances (132). Similarly, removal of 94% of the sialic acid from human luteinizing hormone leads to a marked loss in biological activity (133). The intact carbohydrate moiety of fibrinogen is essential for its role in blood-clotting, and patients suffering from dysfibrinogenemia, caused by a deterioration of the carbohydrate moiety of fibrinogen, encounter difficulties in blood coagulation (113). On the other hand, activities of certain enzymes such as serum cholinesterase and glutamyltranspeptidase are unchanged after removal of sialic acid (126). Little progress has been made in studying the biological role of other sugars because of the difficulties involved in their selective and complete removal from the remainder of the molecule.

This "stepwise removal" approach, however, is tantamount to studying the function of a few amino acids that make up the "active center" of an enzyme and assuming that the rest of the molecule is without any function. In view of the qualitative and quantitative differences found in the content and pattern of heterosaccharide units present in a wide variety of glycoproteins participating in many different types of reactions, it will be difficult to assign a common role to their carbohydrate moieties. It may be more reasonable to assume that in many respects heterosaccharides are like the structural amino acids and perhaps, to some extent, help in shaping and maintaining the conformation of a glycoprotein molecule.

Biosynthesis of Glycoproteins

Sites of Plasma Protein Synthesis

The involvement of the liver in the production of plasma proteins has been recognized for a long time. The earlier work in this area has been reviewed by Madden and Whipple (134). A decrease in serum proteins after partial hepatectomy (52), impairment of plasma protein synthesis following the administration of hepatic poisons (135-136), and a considerable reduction or abolition of the stress-induced increases in glycoprotein response after partial hepatectomy (137) provided evidence supporting the liver as the major site of plasma protein synthesis.

The elegant work of Miller and coworkers (138) on the incorporation of radioactive amino acids into serum proteins by the isolated, perfused rat liver clearly demonstrated the dominant role of this organ in serum protein synthesis. The synthesis of all plasma proteins (except the immunoglobulins) by the liver is now well documented (139). Simkin and Jamieson (140) have studied the in vitro synthesis of serum glycoproteins in liver slices and liver microsomes and have proved that labeled amino acid and sugar precursors can be incorporated into an α-globulin fraction which can be liberated by ultrasonic treatment and precipitated with a specific antiserum to α-globulins.

Synthesis of the Protein Moiety

The synthesis of the polypeptide chains of glycoproteins takes place on polysomes in the conventional manner. Introduction of some improved methods for the fractionation of polysomes into free and membrane-bound species (141, 142) has greatly facilitated work on the functional

differentiation between various populations of polysomes. Evidence in favor of the view that membrane-bound and free polysomes synthesize two different families of proteins is gradually increasing. A number of studies indicate that the majority of proteins destined to be exported from the cell are synthesized by membrane-bound polysomes, while non-exportable, intracellular proteins are mainly synthesized by free polysomes. Thus Redman (143) has reported that while most of the serum proteins are synthesized on membrane-bound polysomes, ferritin, a non-exportable protein, is preferentially synthesized on free polysomes. Similarly, Hick *et al.* (144) have concluded that albumin is synthesized largely on membrane-bound polysomes, whereas ferritin is primarily synthesized on free polysomes. Ogata and coworkers (145) have carried out *in vitro* syntheses of serum proteins on isolated free and bound polysomes and by using a specific antiserum have proved that albumin is synthesized exclusively on membrane-bound polysomes. Recent studies by Glaumann (146) indicate that albumin is synthesized on ribosomes attached to the rough endoplasmic reticulum (RER) and is then transported out of the cell *via* the smooth endoplasmic reticulum (SER) and the Golgi complex. Sherr and Uhr (147) have found that IgG is also largely synthesized on membrane-bound polysomes. The same is true for β-lactoglobulin synthesis in the mammary gland (148).

The concept of functional differentiation and division of labor with respect to protein synthesis poses an obvious question as to how the cell benefits from such a mechanism. The attachment of polysomes to the membranes may offer a number of advantages to the cell (149). The transport of proteins synthesized on these polysomes may be greatly

facilitated. The differential synthesis of some proteins on membrane-bound polysomes and that of others on membrane-free polysomes may assist the cell in making a distinction between proteins to be secreted from the cell and those to be retained inside. The attachment of polypeptides, synthesized on membrane-bound polysomes, to the membranes may also facilitate the association of the subunits of a protein. Tata (150) has suggested that another role for ribosome-membrane association may be to achieve a "topographical segregation" of different groups of ribosomes synthesizing different types of proteins. It has also been suggested that the membranes to which ribosomes are attached may serve to protect mRNA and/or ribosomes from rapid degradation (151).

In accordance with the "functional differentiation" concept, the biosynthesis of plasma glycoproteins would be an exclusive function of membrane-bound polysomes. Substantial evidence indicates that this indeed is true for a number of glycoproteins studied so far (143, 152-154).

The stress-induced increase in the rate of labeled amino acid incorporation into plasma proteins and liver proteins has been observed both *in vivo* (155, 156) and *in vitro* (157-159). Both the microsomes, as well as the cell sap, from the stressed animals showed a higher activity than those from normal controls, but the microsomes were considerably more active than the cell sap (160). Maximum stimulation was obtained 18-24 hours after injury. Since the response to trauma was abolished by puromycin or actinomycin D when administered at the time of injury, Chandler and Neuhaus (161) suggested that the increase in serum proteins in response to stress was a consequence of an increase in mRNA, possibly

due to an increase in its rate of synthesis. A similar two to three-fold increase in amino acid incorporating ability of microsomes and ribosomes isolated from the regenerating liver has been observed by a number of investigators (162-166) with a maximum at 40-48 hours following operation.

Thus, a marked increase in the activities of polysomes isolated from livers of animals subjected to injury and partial hepatectomy seems to be well documented. Whether this increase in activity is a property of membrane-bound polysomes, or free polysomes, or both, still remains to be investigated.

Synthesis of the Carbohydrate Moiety

In contrast to the nucleic acid-directed synthesis of the polypeptide backbone, the synthesis of the oligosaccharide units of glycoproteins is directed only by enzymes. Research in the area of glycoprotein biosynthesis has made considerable progress during the last decade and most of this work has been discussed recently in a number of review articles (58, 139, 167-173).

Initial work on the incorporation of labeled sugar precursors into serum glycoproteins using whole animals (174, 175), isolated, perfused livers (176, 177) and tissue slices (178, 179) led to the identification of the endoplasmic reticulum (microsomes) as the subcellular site of glycosylation (180-182). Most of the sugars added were recovered in the deoxycholate-soluble fractions of liver microsomes, including the RER and SER (183). Simkin and Jamieson (184) injected labeled leucine and glucosamine into guinea-pigs and recovered the label in an α-globulin fraction released from the liver microsomes by ultrasonic treatment. Similar results were obtained when they studied the

in vitro incorporation of N-acetylglucosamine into protein material of isolated liver microsomes (185-186). They noticed, however, that the heterosaccharide units of glycoproteins synthesized *in vitro* were incomplete. The terminal sialic acid residues in particular were lacking.

Li, *et al.* (187) isolated two radioactive glycopeptides from a deoxycholate-soluble fraction of rat liver microsomes which were rich in glucosamine and mannose, but contained little galactose or sialic acid. Based on these findings they concluded that it was primarily the inner cores of the heterosaccharide chains which are synthesized in the microsomes. Lawford and Schachter (153) have presented evidence indicating that the addition of the initial glucosamine residues occurs while the polypeptide chains are being synthesized on membrane-bound polysomes. Further hexosamine residues are added while the polypeptides traverse the channels of the RER and SER on their way out of the cell. Sialic acid is added at the final stages of this synthetic process. Kern and coworkers (188-190) have studied the incorporation of labeled amino acid and sugar precursors into immunoglobulins by lymph node cells and have also concluded that microsomes are the primary site of glycoprotein synthesis. The same conclusions have been drawn by Uhr and coworkers (191, 192) from their work on immunoglobulin synthesis by mouse myeloma cells. Both Kern and colleagues and Uhr and coworkers have suggested that the initial glucosamine residues are added to the nascent polypeptide chains, while galactose and subsequent glucosamine residues are added after the chains are released from the ribosomes. The consensus of opinion now seems to favor the view that the first glucosamine residue is attached to the polypeptide as it is being synthesized and while it is

still attached to the polyribosomes (193-196).

The monosaccharides are not incorporated directly into glycoproteins as such, but are first converted to activated intermediates in the form of sugar nucleotides which act as sugar donors in the enzymatic process of heterosaccharide chain elongation (139, 197). Such intermediates, synthesized in vivo from glucose, include UDP-N-acetylglucosamine, UDP-N-acetylgalactosamine, UDP-glucose, UDP-galactose, GDP-mannose, GDP- fucose, and CMP-sialic acid. The use of these activated monosaccharides in in vitro systems has permitted the identification and characterization of various glycosyltransferases bound to the membranes of the ER (172, 198-206). These enzymes exhibit marked specificities and each monosaccharide is transferred to the growing oligosaccharide chain by a separate transferase. Consequently, there should be at least one and possibly more than one enzyme (to account for positional specificity) for each sugar found in the carbohydrate moieties of various glycoproteins.

Another contribution that made further localization of glycosyltransferases possible was Dallner's introduction of an improved method for the subfractionation of microsomes into rough and smooth forms (207, 208). The use of this method by a number of investigators (209-220) has resulted in the identification of glucosaminyl- and mannosyltransferase activities in the rough and glucosaminyl- and galactosyltransferase activities in the smooth microsomes.

Recent progress in the methodology of isolating intact Golgi membranes (221-225) has revealed that in these organelles most of the peripheral sugar residues are added, and the synthesis of glycoprotein

molecules completed, before secretion. The Golgi membranes contain glucosaminyl-, galactosyl-, fucosyl-, and sialyltransferase activities (226-230), but little or no mannosyltransferase activity has been detected.

Many of these membrane-bound transferases have been solubilized and purified several-fold and the optimal conditions for their maximal activity have been settled in a number of cases (231-240). In addition to unfinished endogenous glycoproteins, the enzymes can also utilize exogenous glycoproteins as their substrates, provided a suitable number of sugar residues is first removed sequentially from the non-reducing end of the oligosaccharide chains (227, 231).

Several lines of evidence (241-245) indicate that the in vivo transfer of monosaccharides from sugar nucleotides to the endogenous glycoprotein acceptors involves lipid intermediates. Since the sugar nucleotides are present in the aqueous cytoplasm, whereas glycoproteins undergoing synthesis are bound to and transported through the lipid-containing membranes of the ER, the idea of an intermediate synthesis of glycolipids capable of transferring sugar residues from a hydrophilic to a hydrophobic environment seems to be highly attractive. Although incorporation of monosaccharides into glycolipids is well documented (241-245) the nature and function of these intermediates is still an open question. Consequently, more work needs to be done to support the "lipid intermediate hypothesis" in the biosynthesis of glycoproteins.

In the light of the evidence currently available, the following general scheme for the biosynthesis of glycoproteins can be visualized.

The apoprotein part of a glycoprotein molecule is synthesized

on membrane-bound polysomes by the conventional template mechanism of protein synthesis. As soon as the polypeptide chains accumulate sufficient amino acids to acquire a particular conformation recognizable by a transferase, the initiation of glycosylation can occur with the addition of the innermost N-acetylglucosamine residue while the polypeptide is still bound to the ribosomes. This is followed by the addition of mannose residues which could either take place immediately before, or more probably after, the release of the polypeptide from polysomes. Subsequent addition of mannose and N-acetylglucosamine residues results in the formation of a branched oligosaccharide core. The addition of a few more mannose and subsequent N-acetylglucosamine residues continues as the incomplete glycoprotein molecules migrate through the membranes of the SER towards the Golgi complex. The addition of galactose might start in the membranes of the SER, but most of the peripheral sugars including N-acetylglucosamine, galactose, fucose and sialic acid seem to be largely incorporated in the membranes of the Golgi complex. There is no clear line of demarcation between membranes of the SER and the Golgi complex. In fact, the Golgi complex may be considered an extension and a derivative of the SER. Thus some of the enzymatic activities are shared by both, but are apparently concentrated in the Golgi complex. The addition of the peripheral sugars, galactose and sialic acid or fucose, completes the synthesis of the carbohydrate side chains and the completed glycoprotein is now enclosed by a Golgi-derived membrane to form a vesicle. This vesicle then migrates, or is transported, through the cytoplasm to the plasma membrane. At this point the vesicular membrane and the plasma membrane merge, releasing the contents of the vesicle to the extracellular

environment.

This scheme of sequential addition of different sugars at different subcellular sites, which is largely based on *in vitro* studies, is also supported by *in vivo* studies as revealed by radioautography (246, 247).

Aims and Objectives

The preceding discussion on the various aspects of glycoprotein synthesis permits the following conclusions: The alterations in the levels of many plasma proteins in response to various types of physical and pathological stress seem to be a manifestation of the same general phenomenon. No matter how the stress stimulus is applied, the resultant increases in plasma levels of glycoproteins are strikingly similar. This is probably because these changes are produced by the same kind of mechanisms. Hormones are certainly important in inducing the stress response but their precise mechanism of action is still not clear.

Although the present state of knowledge of the biological role of individual plasma proteins still does not permit any reasonable evaluation of the true significance of the changes observed after stress, recent progress in elucidating the mechanisms of glycoprotein biosynthesis can serve as a basis for studying the mechanisms of their regulation —— an area equally important and interesting but not sufficiently explored. An understanding of the mechanisms of glycoprotein regulation may lead to a better comprehension of the significance of the changes in the levels of individual plasma proteins. Most studies in this context have been centered around the regulation of the synthesis of the apoprotein part of glycoproteins. Although the regulation of the polypeptide

synthesis is, of course, very important, studies restricted to this portion only are incomplete and are open to many questions. For instance, is the synthesis of the carbohydrate moiety of glycoproteins also regulated along with the protein moiety? Is there a concomitant increase in the synthesis of glycosyltransferases or are they already present in adequate amounts? Is there an increase in the membrane components of the cell to bind increased quantities of glycoproteins undergoing synthesis, or is the rate of glycoprotein transport in the cell enhanced? It was partly to answer these and other such questions that this work was undertaken.

Although stress in various forms has been shown to cause marked stimulation of monosaccharide incorporation into plasma proteins in vivo (248), no one has looked into the possibility of the regulation of the synthesis of oligosaccharide chains of glycoproteins at the subcellular or enzyme level. The successful demonstration by Bley and Chandler (249) that the enzyme responsible for the in vivo synthesis of glucosamine-6-phosphate (L-glutamine-D-fructose-6-phosphate aminotransferase) is induced after injury and partial hepatectomy prompted the undertaking of the present work on the regulation of glycoprotein synthesis at the level of glycosylation. The effects of injury and partial hepatectomy on the activities of glucosaminyl-, galactosyl-, and mannosyltransferase activities were studied and the results of these investigations are reported in this dissertation. Because of the growing evidence that there are functional differences between various populations of polysomes, a study on the effect of injury and partial hepatectomy on the activities and the distributions of membrane-free and membrane-bound polysomes was

also undertaken and the results are reported in this work.

Injury and partial hepatectomy were selected as model systems for studying the regulation of glycoprotein biosynthesis throughout this work. Although the acute-phase response can be produced by a variety of treatments including experimental infection, scalding, subcutaneous injections of corrosive agents, and bone fractures, simple laparotomy was chosen because of its relative aseptic nature, reproducibility, semi-quantitative control and many other factors discussed by Chandler (250). Partial hepatectomy offered a different, more drastic, but similar model system; one which has been studied extensively except in the area of glycoprotein synthesis and regulation.

CHAPTER II

MATERIALS AND METHODS

Materials

Male albino rats, 50-60 days old, weighing from 225 to 250 g, were purchased from the Holtzman Company, Madison, Wisconsin. The animals were fed standard laboratory chow and water ad libitum for at least one week prior to use.

UDP-N-acetylglucosamine-1-^{14}C (42 mC/mmole); UDP-galactose-^{14}C [D-galactose-^{14}C (U.L.)], (252 mC/mmole); GDP-mannose-^{14}C [D-mannose-^{14}C (U.L.)], (155 mC/mmole); L-leucine-^{14}C (U.L.), (255 mC/mmole); and L-phenylalanine-^{14}C (U.L.), (366 mC/mmole) were purchased from New England Nuclear Corporation.

Unlabeled UDP-N-acetylglucosamine, ATP and GTP were obtained from Sigma Chemical Company, and UDP-galactose, GDP-mannose, phosphoenolpyruvate and pyruvate kinase were purchased from Calbiochem.

"Ultrapure" sucrose, L-amino acids, and CsCl were purchased from Mann Research Laboratories. Dextran 500 was obtained from Pharmacia and Triton X-100, from Rohm and Haas. Fetal calf serum was purchased from GIBCO. Human plasma was obtained from the Oklahoma Medical Research Foundation (Cancer Section). Chromatographically-purified β-galactosidase (E.coli) was purchased from Worthington Enzyme Corporation.

Hyamine hydroxide, PPO, and dimethyl POPOP were purchased from Packard Instrument Company.

Methods

Surgical Techniques

Hepatectomies were performed by the method of Higgins and Anderson (251). Injuries were produced by laparotomy (sham hepatectomy). The procedure was that used for partial hepatectomy, but stopped short of the manipulation and ligation of the liver lobes.

Preparation of Liver Homogenates

The animals were fasted for 18 to 20 hours prior to sacrifice in order to deplete the liver of its glycogen content. Injured animals were sacrificed 24 hours after surgery and partially hepatectomized animals 48 hours after surgery, except where indicated. The animals were anesthetized with ether and their livers were perfused *in situ* through the portal vein with 50 ml of 0.15 M ice-cold saline solution. The livers were then removed, rinsed with a small portion of homogenizing medium, blotted on absorbent paper, weighed and put into 10 ml of ice-cold homogenizing medium. All further steps prior to incubation were carried out at 0-4° and all apparatus and solutions used thereafter were prechilled to 0-4°. The livers were minced with sharp scissors, transferred to a homogenizer, and the total volume of the homogenization medium was brought to 3 volumes (w/v).

Gentle homogenization was performed in a loose-fitting Potter-Elvehjem Teflon-glass tissue homogenizer (Arthur Thomas Co., Type C) at a speed of 1500 rpm, using four, complete up and down strokes for livers

from control and injured animals and six complete strokes for livers from partially hepatectomized animals. Livers from partially hepatectomized rats required longer homogenization to obtain adequate yields of subcellular fractions. In most of the experiments, homogenates from 3-4 livers removed from animals subjected to similar treatment were pooled to obtain sufficient amounts of the required fractions.

Isolation of Free and Bound Polysomes

Free and membrane-bound polysomes were isolated according to the method of Blobel and Potter (252). Livers were homogenized in 0.25 M sucrose, buffered with 0.05 M Tris-maleate, pH 7.6, containing 0.005 M $MgCl_2$, and 0.025 M KCl (TMK). Postmitochondrial supernatants (5 ml) from pooled liver homogenates were layered on top of two-step discontinuous sucrose density gradients composed of 4 ml each of 0.5 M and 2.0 M sucrose. All sucrose solutions were buffered with TMK. The gradients were centrifuged at 40,000 rpm in an SW 40 Ti rotor for 24 hours. Free polysomes sedimented through the 2.0 M sucrose layer and were collected as a pellet. Membrane-bound polysomes formed a band at the interface between 0.5 M and 2.0 M sucrose layers and were withdrawn with a pipette. Both free and bound polysomes were treated for 15-20 minutes with sodium deoxycholate and Triton X-100 (final concentrations 1% and 4%, respectively) to eliminate contaminating membranes. The resulting polysomal suspensions were layered on top of the two-step gradient described above and centrifuged for another 24 hours. The supernatants were discarded and the pellets were rinsed and suspended in 0.05 M Tris-maleate, pH 7.0, to give a final concentration of 8-12 mg of protein per ml for use in the amino acid incorporation assays.

Isolation of Total Microsomes

Microsomes were isolated and fractionated by the method of Dallner (207), using 0.25 M sucrose buffered with 0.01 M Tris-maleate, pH 7.4, as the homogenization medium.

The homogenates were centrifuged at 10,000 x g for 10 minutes in a Sorvall RC-2B refrigerated centrifuge using an SS-34 rotor and 50 ml capacity polypropylene centrifuge tubes. The supernatant fluid was decanted and passed through four layers of cheese-cloth to remove lipids, and the postmitochondrial supernatant was centrifuged at 105,000 x g for 60 minutes in a Beckman Ultracentrifuge using the 50-Ti rotor. The resulting supernatant was discarded and the surface of the microsomal pellet was rinsed carefully with 0.05 M Tris-maleate, pH 7.0. The pellets were gently resuspended in the same buffer with the help of a manually operated Teflon-glass homogenizer. The final volumes were adjusted to obtain a concentration of 15-20 mg protein per ml. These suspensions were used in the sugar incorporation assays.

Fractionation into Rough and Smooth Microsomes

All sucrose solutions used in this procedure were buffered with 0.01 M Tris-maleate, pH 7.4. The microsomal pellets obtained above were gently resuspended in 0.25 M sucrose and enough CsCl was added from a 1.5 M solution to give a final concentration of 15 mM. Six and one-half ml of this suspension was layered over 6.5 ml of 1.3 M sucrose, also made 15 mM with respect to CsCl. The gradients were centrifuged in a 50-Ti rotor at 165,000 x g for 3 hours. Rough microsomes sedimented through the 1.3 M sucrose layer and were obtained as a pellet. Smooth microsomes were withdrawn carefully with a pipette from the interface between the

two layers, diluted 1:1 with 0.25 M sucrose, and sedimented by centrifugation at 105,000 x g for 60 minutes. The pellets were suspended in 0.05 M Tris-maleate, pH 7.0, to give 16-32 mg of protein per ml buffer. These suspensions were used in the sugar incorporation assays.

Isolation of Golgi-Rich Fraction

The Golgi-rich fractions were isolated by a modification of a method developed by Morré and coworkers (221). Since starved rats were reported to yield relatively poor preparations of Golgi fractions (253), unstarved rats were used to isolate these fractions. Livers were homogenized in a medium consisting of 0.05 M Tris-maleate, pH 7.0, 0.5 M sucrose, 1% dextran, and 0.005 M $MgCl_2$. Homogenates from 4-5 animals were pooled and centrifuged at 1,500 x g for 15 minutes in a Sorvall refrigerated centrifuge to sediment the cell debris and most of the nuclei. The pellets were discarded and the supernatants, carefully removed by decantation, were centrifuged again at 5,000 x g for 20 minutes. This time the supernatants were discarded and the pellets were gently homogenized into 5 ml of 1.5 M sucrose. The suspensions were transferred to 33 ml capacity cellulose nitrate-tubes. Discontinuous gradients were made by layering 15 ml of 1.25 M sucrose over the suspensions and then 12 ml of 0.5 M sucrose on the top. All sucrose solutions were prepared in a mixture consisting of 0.05 M Tris-maleate, pH 7.0, 1.0% Dextran 500 and 0.005 M $MgCl_2$. The gradients were centrifuged in an SW 25.1 rotor at 25,000 rpm for 150 minutes using a Beckmen Model L2-65B ultracentrifuge. The Golgi membranes formed a cream-colored band at the interface between the 1.25 M and 0.5 M sucrose layers. These were withdrawn by means of a Beckman Fraction Recovery System.

After diluting 1:1 with 0.15 M saline solution the Golgi fractions were sedimented by centrifugation at 105,000 x g for 60 minutes. In order to reduce contamination of the fractions by plasma membranes and endoplasmic reticulum, the pellets were resuspended in the homogenization medium and sedimented at 2,000 x g. This step was repeated 2-3 times. The final pellets were carefully rinsed with 0.05 M Tris-maleate, pH 7.0, and were suspended in the same buffer to a final concentration of 2-6 mg protein per ml. These suspensions were used in the sugar incorporation assays.

Incorporation of Amino Acids into Polysomes

The incubation mixture for amino acid incorporation assays contained 175 µmoles of Tris-maleate, pH 7.4; 0.25 µmoles of GTP; 1.0 µmole of ATP; 10 µmoles of phosphoenolpyruvate; 100 µg of pyruvate kinase; 35 µmoles of KCl; 2.0 µmoles of $MgCl_2$; 0.2 µmoles of a mixture of 18 L-amino acids; 0.5 µC of L-leucine-^{14}C; 0.5 µC of L-phenylalanine-^{14}C; 0.25 ml of polysomal suspension containing 2-5 mg of protein; and 0.5 ml of the cell sap, in a total volume of 1.0 ml. The mixtures were incubated for 30 minutes at 37° and precipitated by the addition of 3 ml cold 5% TCA solution. These samples were stored overnight at 0-4° to effect complete precipitation of protein. The protein precipitates were heated for 10 minutes at 90° in 5% TCA to release non-protein-bound label, cooled, centrifuged in a clinical centrifuge, and processed for liquid scintillation counting.

Incorporation of Monosaccharides into Microsomal Fractions

Incubation mixtures for the <u>in vitro</u> incorporation of sugars

contained, in a total volume of 100 µl, 0.5 µmoles of Tris-maleate, pH 7.0; 1 µmole of $MnCl_2$; 0.5 µmoles of $MgCl_2$; 0.1 µmole of KCl; 0.5 µmoles of EDTA; Triton X-100 to a final concentration of 1%, where indicated; 20,000 cpm of sugar nucleotide, labeled with ^{14}C in the sugar moiety; 0.2 µmoles of two other unlabeled sugar nucleotides, where indicated; and 40-60 µl of microsomal suspension containing 0.8-1.5 mg of protein. Assays were carried out in 15 ml capacity clinical centrifuge tubes in a shaking water bath at 37° for one hour. The reactions were stopped by the addition of 3 ml of ice-cold 5% TCA or 3 ml of 5% phosphotungstic acid solution in 2 N HCl and the samples were stored overnight to effect complete precipitation of protein. The tubes were heated at 90° for 10 minutes to release labeled sugars not actually incorporated into protein. The samples were cooled and protein precipitates were collected by centrifugation in a clinical centrifuge and processed for counting.

Incorporation of Monosaccharides into Golgi-Rich Fractions

The complete incubation mixture for monosaccharide incorporation into Golgi-rich fractions contained, in a total volume of 100 µl, 4 µmoles of Tris-maleate, pH 7.0; 1.0 µmole of $MnCl_2$; 0.5 µmoles of $MgCl_2$; 0.5 µmoles of EDTA; Triton X-100 to a final concentration of 1%, where indicated; 20,000 cpm of the ^{14}C-labeled sugar nucleotide; 0.2 µmoles of two other unlabeled sugar nucleotides, where indicated; and 40-60 µl of Golgi suspension containing 0.2-0.6 mg protein. Incubations were carried out as described in the previous section.

Preparation of Samples for Liquid Scintillation Counting

The protein precipitates were washed 3 times each with 3 ml of

cold 5% TCA or phosphotungstic acid solution and once with 3 ml of 95% ethanol. The pellets were then extracted with 3 ml of chloroform-methanol (2:1) and finally washed with 3 ml of diethyl ether, dried and dissolved in 2 ml of 0.2 N NaOH by warming to 80° in a water bath. A measured portion of the NaOH solution was removed for determination of protein content and that remaining was digested overnight with 0.5 ml of Hyamine hydroxide in scintillation vials placed in a 55° water bath. The digested samples were mixed with 10-12 ml of the "XDC" scintillation mixture of Bruno and Christian (254) and were counted in a liquid scintillation counter. All samples were corrected to 100% efficiency by the use of internal or external standards.

Extraction of Glycolipids

The TCA precipitates obtained at the end of the reaction, as described above, were washed 3 times each with 3 ml of cold 5% TCA solution and once with 3 ml of 95% ethanol. The pellets were then extracted twice each with 5 ml of chloroform-methanol (2:1) for 15 minutes. The extracts were combined and washed with 0.15 M saline solution, dried in a stream of hot air and counted directly as described above.

Galactosyltransferase (Lactosamine Synthetase) Assay

Galactosyltransferase activities were measured by the method of Babad and Hassid (255). Liver homogenates were diluted 10-fold with 0.5% Triton X-100 and filtered through four layers of cheese-cloth. One-tenth ml of filtrate was used in each assay. The assay mixture contained 4 μmoles of Tris-maleate, pH 7.0; 0.5 μmoles of $MgCl_2$; 1.0 μmole of $MnCl_2$; 2 μmoles of mercaptoethanol; 1 μmole of N-acetylglucosamine; 0.3-0.6 mg of enzyme protein, and 12,000 cpm of UDP-galactose-^{14}C in a

total volume of 0.15 ml. Incubations were carried out in a 37° shaking water bath and the reactions were stopped after 5 minutes by adding 20 µl of 0.25 M EDTA neutralized with NaOH. These mixtures were applied to 1.5 ml (6 cm x 0.6 cm) columns of AG1-X2 resin (200-400 mesh, chloride form) prepared in distilled water. N-acetyllactosamine was eluted from the columns by 3 x 0.5 ml washes of distilled water and collected in scintillation vials. After adding 12 ml of the scintillation mixture, the samples were counted directly. All assays were corrected for the non-specific hydrolysis of UDP-galactose-^{14}C by hydrolases present in the homogenates. This correction was obtained by incubating and processing duplicate reaction mixtures in which N-acetylglucosamine was omitted. The counts released as pure galactose were then subtracted from the total counts obtained from the corresponding complete reaction mixture.

Electron Microscopy

Golgi pellets were fixed for one hour in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, at 4° and left overnight in the buffer. The samples were rinsed and postfixed for two hours in 1% OsO_4 in 0.1 M cacodylate buffer, pH 7.2, at 4°. Dehydration was carried out in a graded series of ethanol concentrations, followed by propylene oxide. Specimens were embedded in Maraglas and silver sections were cut with a diamond knife on a Porter-Blum MT-1 microtome. The sections were mounted on uncoated copper grids and were stained with saturated, acidic uranyl acetate at 60° for 15 minutes followed by lead citrate for 8 minutes at room temperature. Electron micrographs were obtained with an RCA-EMU 3F electron microscope operated at 50 kv.

Liver cells were fixed and examined in exactly the same manner

except that primary fixation was performed in 4% glutaraldehyde.

Analytical Procedures

Protein was precipitated with 5% TCA solution, washed with ethanol and finally with ether. The precipitates were dissolved in 0.2 N NaOH and protein was determined by the method of Lowry et al. (256), or by the method of Gornall et al. (257), using bovine serum albumin as the standard. Ribonucleic acid was determined by the method of Schmidt and Thannhauser as modified by Munro and Fleck (258). Deoxyribonucleic acid (DNA) was determined by the method of Burton (259). Phosphorus was measured according to the method of Fiske and SubbaRow (260). Phosphorus readings were multiplied by 25 to obtain the amount of phospholipids.

Statistical Analysis

Whenever possible and appropriate, statistical analyses were carried out on paired observations using the standard two-sided "student's t test." Paired comparisons (i.e., control and experimental observations obtained in the same experiments were considered paired) were employed because of large variations observed between individual sets of experiments. In other instances, where appropriate and valid, comparisons were based on group analysis.

Differences between two values were considered significant only if the P values were less than 0.05.

CHAPTER III

RESULTS

Effects of Injury and Partial Hepatectomy on Membrane-Bound and Free Polysomes

Injury and partial hepatectomy stimulate hepatic RNA synthesis and result in a shift in the polysomal profiles towards heavier aggregates. It has been suggested that these changes are indicative of an increase in the rate of polysome formation (160). Since injury also stimulates glycoprotein synthesis, which is primarily a function of membrane-bound polysomes (152-154), an increase in the population of liver polysomes as a result of injury should be reflected mainly or solely in the membrane-bound polysomes. The amount of free polysomes, on the other hand, may or may not be changed to any significant extent since the synthesis of most non-exportable, intracellular proteins does not seem to be stimulated by stress as much as that of secretory proteins. Consequently, the ratio of membrane-bound to free polysomes in the liver should be significantly increased in stressed animals. In the regenerating liver, however, there is an increase in the rate of synthesis of intracellular proteins as well as of exportable, extracellular proteins, and, therefore, one might expect a different picture. The results of experiments designed to study the effects of injury and partial hepatectomy on the distribution of polysomes in the rat liver are shown in Table 1.

TABLE 1

EFFECT OF INJURY AND PARTIAL HEPATECTOMY ON THE RELATIVE DISTRIBUTION OF FREE AND MEMBRANE-BOUND POLYSOMES IN RAT LIVER

Polysomes	Control	Injury	Partial Hepatectomy
Free Polysomes		mg RNA/g liver	
Mean ± s.e.m.	1.77 ± 0.06	1.51 ± 0.08	2.53 ± 0.16
No. of Observations	9	8	8
Difference (P)	-----	<0.02	<0.001
Bound Polysomes			
Mean ± s.e.m.	0.97 ± 0.10	1.72 ± 0.11	0.93 ± 0.14
No. of Observations	9	8	8
Difference (P)	-----	<0.001	N.S.
Bound/Free Polysomes			
Mean ± s.e.m.	0.55 ± 0.04	1.17 ± 0.04	0.36 ± 0.05
Difference (P)	-----	<0.001	<0.01

Polysomes were isolated essentially as described in Methods, except that centrifugation was carried out in a 50 Ti rotor at 45,000 rpm, and the pellets were suspended in distilled water for RNA determinations.

Statistical analysis was based on non-paired comparison of groups. N.S. refers to "not significant."

Injury caused a two-fold increase in the membrane-bound to free polysome ratio in the liver. This was due primarily to a marked increase in the amount of membrane-bound polysomes. At the same time there appeared to be a small, but significant, decrease in the quantity of free polysomes.

Partial hepatectomy, on the other hand, resulted in a considerable decrease in the ratio of membrane-bound to free polysomes due to an increase in the amount of free polysomes with little or no change in the quantity of membrane-bound polysomes. Because regenerating livers were subjected to longer periods of homogenization, the comparison of the absolute amounts of free and membrane-bound polysomes with those of control livers must be made with caution, and for this reason, the ratios of membrane-bound to free polysomes were considered to be a more reliable parameter.

Free and membrane-bound polysomes isolated from animals subjected to injury or partial hepatectomy had amino acid incorporating abilities comparable to those isolated from normal controls (Table 2). The incorporating abilities of free polysomes and bound polysomes were also equivalent. This is in agreement with the results of other investigators (142, 261). Failure to detect an effect of injury or partial hepatectomy on the activity of the polysomes was surprising in the light of the findings of Liu and Neuhaus (160) and Majumdar et al. (54) that the amino acid incorporating ability of the microsomes and polysomal aggregation were increased following these treatments. The isolation of polysomes by the method of Blobel and Potter (141) required treatment with detergents like sodium deoxycholate and Triton X-100 to free the

TABLE 2

EFFECT OF INJURY AND PARTIAL HEPATECTOMY ON CELL-FREE AMINO ACID INCORPORATION BY FREE AND MEMBRANE-BOUND POLYSOMES

Polysomes	Control	Injury	Partial Hepatectomy
		dpm/mg protein/30 min.	
Free Polysomes			
	1218	1230	1238
	1200	1184	1216
	2192	2360	2450
	2337	2527	2599
	3891	5021	5041
	6568	6191	6530
Mean ± s.e.m.	2901 ± 836	3086 ± 843	3179 ± 879
Difference (P)	----	N.S.	N.S.
Bound Polysomes			
	1510	1106	1342
	1136	1822	----
	2166	2702	2220
	2353	2870	2206
	4607	5699	3577
	6283	6117	6316
Mean ± s.e.m.	3009 ± 820	3386 ± 840	3132 ± 873
Difference (P)	----	N.S.	N.S.

The assays were carried out as described in Methods. The cell sap used in all instances was derived from control livers in order to obtain a uniform effect of the supernatant factors.

Statistical analysis was based on paired observations; N.S. refers to "not significant."

polysomes from the membranes. Blobel and Potter (262) and Ogata and coworkers (263) found that this treatment released ribonuclease and caused marked degradation of polysomes to disomes and monosomes with a corresponding decrease in amino acid incorporating ability.

Table 3 shows the results of an experiment designed to test the possibility that detergent treatment masked an increase in polysomal activity following injury and partial hepatectomy. It is clear that both free and membrane-bound polysomes isolated without detergent treatment exhibited an increase in amino acid incorporating ability after partial hepatectomy. This time the bound polysomes reached much lower specific activities than the free polysomes because of the high content of protein in the membranes. Treatment with detergents lowered the activities of the free polysomes drastically and brought all preparations to the same limiting value. Thus it appears that injury and partial hepatectomy did increase the protein synthesizing capacity of free and bound polysomes but this increase could not be detected because of the method used for their isolation. Another possible explanation could be that injury and partial hepatectomy caused an increase in the amounts of polysomes without any significant change in their activity.

No appreciable differences were detected in the RNA to protein ratios of polysomes between control and experimental preparations (Table 4). The RNA/protein ratio was slightly higher in free polysomes when compared to membrane-bound polysomes.

Effects of Injury and Partial Hepatectomy on Glycosylation

Since microsomes are comprised of ribosomes, the sites of assembly of polypeptides, and of membranes, the location of many of the

TABLE 3

EFFECT OF INJURY AND PARTIAL HEPATECTOMY ON CELL-FREE AMINO ACID INCORPORATION BY FREE AND MEMBRANE-BOUND POLYSOMES PREPARED WITH AND WITHOUT DETERGENT TREATMENT

Polysomes	Control	Injury	Partial Hepatectomy
		dpm/mg protein/30 min.	
Free Polysomes			
Without Detergent	158,085	163,947	243,429
With Detergent	18,278	17,218	28,783
Bound Polysomes			
Without Detergent	19,946	25,428	28,456
With Detergent	18,933	------	18,728

The assays were carried out essentially as described in Methods. The polysomal preparations without detergent treatment were used one day following the sacrifice of the animals (24 hours centrifugation), while those with detergent treatment were used two days following the sacrifice of the animals (48 hours centrifugation). In this experiment a complete, tritiated amino acid mixture was used for incorporation. Each value represents the pooled livers of 4 animals in all cases.

TABLE 4

EFFECT OF INJURY AND PARTIAL HEPATECTOMY ON
RNA TO PROTEIN RATIOS OF POLYSOMES

Polysomes	Control	Injury	Partial Hepatectomy
		RNA/protein	
Free Polysomes			
Mean ± s.e.m.	0.78 ± 0.024	0.75 ± 0.027	0.78 ± 0.030
No. of Observations	10	10	10
Difference (P)	----	N.S.	N.S.
Bound Polysomes			
Mean ± s.e.m.	0.66 ± 0.029	0.69 ± 0.024	0.64 ± 0.023
No. of Observations	11	11	11
Difference (P)	----	N.S.	N.S.

The differences between the RNA/protein ratios for free and membrane-bound polysomes were significant ($p < 0.005$) for preparations from control and partially hepatectomized animals but not for injured animals.

glycosyltransferases, it seemed logical to begin the study of the effects of injury and partial hepatectomy on the glycosylation process with this fraction. The glycosyltransferases studied were those for N-acetylglucosamine, galactose, and mannose.

Total Microsomes

A significant increase in the incorporation of N-acetylglucosamine into the total microsomal fraction was seen after injury but not after partial hepatectomy (Table 5). The pattern of galactose incorporation into microsomes was similar to that for N-acetylglucosamine. Mannose incorporation, however, was much greater after partial hepatectomy (about 100%) than after injury (about 40%). It is interesting to note that the degree of stimulation caused by injury was about the same (40%) for all three sugars.

Postmicrosomal Fractions

Reports by Wagner and Cynkin (209) and by Eylar (264) indicated that postmicrosomal particles, which are presumably derived from the SER (265), had much higher glucosaminyltransferase activities than microsomes. Accordingly, several experiments were performed to see if injury and partial hepatectomy had an effect on N-acetylglucosamine incorporation into postmicrosomal particles. The results of these experiments are shown in Table 6.

Injury had no effect, but partial hepatectomy resulted in a striking reduction in glucosamine incorporation into the postmicrosomal fraction. The significance of this reduction after partial hepatectomy is unknown. Although these results are presented on a mg protein basis

TABLE 5

TRANSFER OF LABELED MONOSACCHARIDES FROM SUGAR NUCLEOTIDES TO ENDOGENOUS GLYCOPROTEIN ACCEPTORS ASSOCIATED WITH TOTAL MICROSOMES

Sugar Nucleotide	Control	Injury	Partial Hepatectomy
		dpm/mg protein/hour	
UDP-N-acetyl-glucosamine-^{14}C	156	282	194
	177	364	214
	333	392	414
	394	468	386
Mean ± s.e.m.	265 ± 68	377 ± 58	302 ± 57
Difference (P)	----	<0.05	N.S.
GDP-mannose-^{14}C	207	376	398
	256	385	554
	294	432	670
	386	452	730
Mean ± s.e.m.	286 ± 38	411 ± 18	588 ± 73
Difference (P)	----	<0.025	<0.025
UDP-galactose-^{14}C	304	417	293
	348	464	500
	454	668	564
	371	449	429
Mean ± s.e.m.	369 ± 32	500 ± 57	446 ± 58
Difference (P)	----	<0.025	N.S.

Statistical analysis was performed on paired observations. Each value represents pooled livers from 3-4 animals in all cases.

TABLE 6

TRANSFER OF N-ACETYLGLUCOSAMINE-^{14}C FROM UDP-N-ACETYLGLUCOSAMINE-^{14}C TO ENDOGENOUS GLYCOPROTEIN ACCEPTORS ASSOCIATED WITH POSTMICROSOMAL FRACTIONS

	Control	Injury	Partial Hepatectomy
		dpm/mg protein/hour	
	562	484	193
	581	508	326
	790	778	810
	1300	1238	481
	1622	1566	594
	1467	1616	337
Mean ± s.e.m.	1055 ± 191	1031 ± 209	437 ± 90
Difference (P)	----	N.S.	<0.025

Postmicrosomal particles were prepared by the method of Siekevitz as modified by Wagner and Cynkin (209). Livers were homogenized in 2.3 volumes of a medium consisting of 0.35 M sucrose, 0.025 M KCl, 0.005 M $MgCl_2$, 0.005 M mercaptoethanol, and 0.05 M Tris maleate, pH 7.5. Nuclei, mitochondria and cell debris were removed by sedimentation at 29,000 x g for 12 minutes. The supernatant was decanted and centrifuged at 105,000 x g for 40 minutes. The microsomal supernatant was withdrawn and the pellet was rinsed with 2 ml of homogenizing medium, with swirling, to remove the loose, gray material at the top. The supernatant and the rinsings were combined and centrifuged at 105,000 x g for 3 hours to yield the postmicrosomal pellet. After rinsing with 0.05 M Tris maleate, pH 7.0, the pellet was suspended in the same buffer and used in the assay.

they should be considered with caution. The decrease seen after partial hepatectomy, for example, could result from differences in the sedimentation properties of the postmicrosomal particles or from their recovery by the swirling technique, rather than from a real change in enzyme activities.

Microsomal Subfractions

The successful demonstration of the stimulation of monosaccharide incorporation into total microsomes by stress led to further work on tracing glycosyltransferase activities in the rough and smooth ER membranes. The results summarized in Table 7 indicate that injury and partial hepatectomy had no significant effect on N-acetylglucosamine or galactose incorporation into rough microsomes. On the other hand, mannose incorporation into rough microsomes was increased two-fold after partial hepatectomy and, although not statistically significant, there seemed to be a consistent increase after injury as well.

With smooth microsomes, the situation was altogether different (Table 8). N-Acetylglucosamine incorporation was increased two-fold after injury, but partial hepatectomy had no effect. Again, galactose incorporation into the smooth microsomal fractions closely followed the pattern of N-acetylglucosamine incorporation. Mannose incorporation into these fractions was stimulated slightly both by injury and partial hepatectomy, but the latter was not statistically significant in these experiments.

Examination of Tables 7 and 8 also shows that smooth microsomes had much higher activities for N-acetylglucosamine and galactose incorporation than did rough microsomes, whereas the converse was true

TABLE 7

TRANSFER OF LABELED MONOSACCHARIDES FROM SUGAR NUCLEOTIDES TO ENDOGENOUS GLYCOPROTEIN ACCEPTORS ASSOCIATED WITH ROUGH MICROSOMES

Sugar Nucleotide	Control	Injury	Partial Hepatectomy
		dpm/mg protein/hour	
UDP-N-acetyl-glucosamine-^{14}C	112	138	126
	138	132	130
	158	226	200
	96	102	104
	100	78	62
	184	200	151
Mean ± s.e.m.	131 ± 14	146 ± 23	129 ± 19
Difference (P)	----	N.S.	N.S.
GDP-mannose-^{14}C	100	112	262
	226	386	582
	202	228	340
	285	491	492
Mean ± s.e.m.	203 ± 39	304 ± 84	412 ± 67
Difference (P)	----	N.S.	<0.025
UDP-galactose-^{14}C	46	32	44
	112	120	154
	98	96	84
	166	210	181
Mean ± s.e.m.	106 ± 25	115 ± 37	116 ± 32
Difference (P)	----	N.S.	N.S.

TABLE 8

TRANSFER OF LABELED MONOSACCHARIDES FROM SUGAR NUCLEOTIDES TO ENDOGENOUS GLYCOPROTEIN ACCEPTORS ASSOCIATED WITH SMOOTH MICROSOMES

Sugar Nucleotide	Control	Injury	Partial Hepatectomy
		dpm/mg protein/hour	
UDP-N-acetyl-glucosamine-^{14}C	328	350	240
	316	550	216
	238	492	290
	230	418	324
	172	516	214
	363	826	389
Mean ± s.e.m.	275 ± 30	525 ± 67	278 ± 28
Difference (P)	----	<0.01	N.S.
GDP-mannose-^{14}C	78	88	106
	76	108	132
	54	80	154
	158	205	142
Mean ± s.e.m.	92 ± 23	120 ± 29	134 ± 10
Difference (P)	----	<0.05	N.S.
UDP-galactose-^{14}C	552	960	372
	538	1354	732
	436	1354	378
	478	883	467
Mean ± s.e.m.	501 ± 27	1138 ± 125	487 ± 84
Difference (P)	----	<0.02	N.S.

for mannose incorporation. These results contradict some previous reports that showed rough microsomes to be more active in N-acetylglucosamine incorporation than smooth microsomes (210, 216). These studies were, therefore, extended further to include the *in vivo* incorporation of glucosamine into microsomal fractions. The results are presented in Table 9. Smooth microsomes were again found to be 2-3 times as active as rough microsomes, supporting our *in vitro* results.

Ribonucleic Acid and Phospholipid Contents of Various Subcellular Fractions

The RNA and phospholipid contents of various cell fractions were determined to see if any changes were caused by injury or partial hepatectomy. The results are presented on a per mg protein and a per mg DNA basis in Table 10.

The only significant change observed was an increase in RNA after partial hepatectomy, reflected chiefly in those fractions containing rough microsomes. The phospholipid/protein ratio was also higher in total microsomes isolated from partially hepatectomized animals as compared to controls but examination of other subcellular fractions did not show any significant differences.

The high RNA and low phospholipid contents of the rough microsomes *versus* the low RNA and high phospholipid contents of the smooth microsomes are indicative of a good separation of the two fractions. Compared to many values reported in the literature the phospholipid/protein ratios are high. This is because these fractions were thoroughly washed with TCA and organic solvents prior to determination of protein content. This treatment removed contaminating serum albumin, a factor

TABLE 9

IN VIVO INCORPORATION OF GLUCOSAMINE-^{3}H INTO GLYCOPROTEINS OF MICROSOMAL SUBFRACTIONS

	Total Microsomes	Rough Microsomes	Smooth Microsomes
		dpm/mg protein	
	6,000	4,645	12,362
	6,130	5,225	12,375
	5,906	4,500	11,753
	6,030	5,410	12,895
	6,112	5,719	13,913
Mean ± s.e.m.	6,036 ± 41	5,100 ± 230	12,659 ± 362

Glucosamine-^{3}H (10 μC/100 g) was injected via the jugular vein and the animals were sacrificed one hour after injection. Microsomes were isolated and the radioactivity measured as described in Methods.

TABLE 10

CHARACTERIZATION OF VARIOUS SUBCELLULAR FRACTIONS

Cell Fraction	Control	Injury	Partial Hepatectomy
		RNA/protein	
Liver Homogenate	0.057 ± 0.003(4)	0.058 ± 0.003(4)	0.072 ± 0.004(4)
	------	N.S.	P=0.025
Total Microsomes	0.191 ± 0.007(7)	0.204 ± 0.009(7)	0.307 ± 0.016(7)
	------	N.S.	P<0.001
Rough Microsomes	0.315 ± 0.015(4)	0.330 ± 0.017(4)	0.410 ± 0.023(4)
	------	N.S.	P<0.02
Smooth Microsomes	0.055 ± 0.013(4)	0.045 ± 0.007(4)	0.063 ± 0.010(4)
	------	N.S.	N.S.
		PL/protein	
Liver Homogenate	0.200 ± 0.004(4)	0.210 ± 0.012(4)	0.200 ± 0.004(4)
	------	N.S.	N.S.
Total Microsomes	0.424 ± 0.010(4)	0.472 ± 0.026(4)	0.616 ± 0.020(4)
	------	N.S.	P<0.001
Rough Microsomes	0.285 ± 0.023(4)	0.315 ± 0.021(4)	0.268 ± 0.029(4)
	------	N.S.	N.S.
Smooth Microsomes	0.493 ± 0.026(4)	0.513 ± 0.015(4)	0.543 ± 0.033(4)
	------	N.S.	N.S.
		RNA/DNA	
Liver Homogenate	4.465 ± 0.170(8)	4.598 ± 0.200(8)	5.260 ± 0.180(8)
	------	N.S.	P<0.01
		PL/DNA	
Liver Homogenate	14.74 ± 1.12(4)	14.69 ± 0.96(4)	14.45 ± 0.58(4)
	------	N.S.	N.S.

Values are expressed as mean ± s.e.m. The numbers in parenthesis refer to the number of observations. P=probability. PL=phospholipids.

taken into account by only a few investigators (266). The values reported here are in excellent agreement with those of Glauman and Dallner (267, 268).

Golgi-Rich Fractions

The Golgi fractions isolated in these experiments exhibited very high glucosaminyl- and galactosyltransferase activities of the order of 20-30 times that exhibited by total microsomes or crude liver homogenates (Table 11). The specific activities of the transferases present in the Golgi-rich fractions may be used as rough estimate of the purity of these fractions (168). The values shown in Table 11 are in good agreement with those reported by others (224-227). Injury or partial hepatectomy caused no changes in the specific activities of glucosaminyl- or galactosyltransferases when compared to each other or to control values. These results indicate that the Golgi-rich fractions isolated from control, injured and partially hepatectomized animals had equal glycosyltransferase specific activities. It is possible, however, though unlikely, that the various Golgi-rich fractions had unequal transferase activities and were also of unequal purity so that fortuitously equal specific activities were obtained.

Electron microscopic examination of intact and isolated Golgi complexes was undertaken to see if injury or partial hepatectomy caused morphological changes in this organelle. Plates 1-3 show electron micrographs of portions of hepatocytes from control, injured and partially hepatectomized animals. The appearance of typical stacks of parallel, flattened cisternae surrounded by vesicular profiles and secretory vesicles mark the Golgi apparatus. The elongated, flattened

TABLE 11

TRANSFER OF LABELED MONOSACCHARIDES FROM SUGAR NUCLEOTIDES TO ENDOGENOUS GLYCOPROTEIN ACCEPTORS ASSOCIATED WITH GOLGI-RICH FRACTIONS

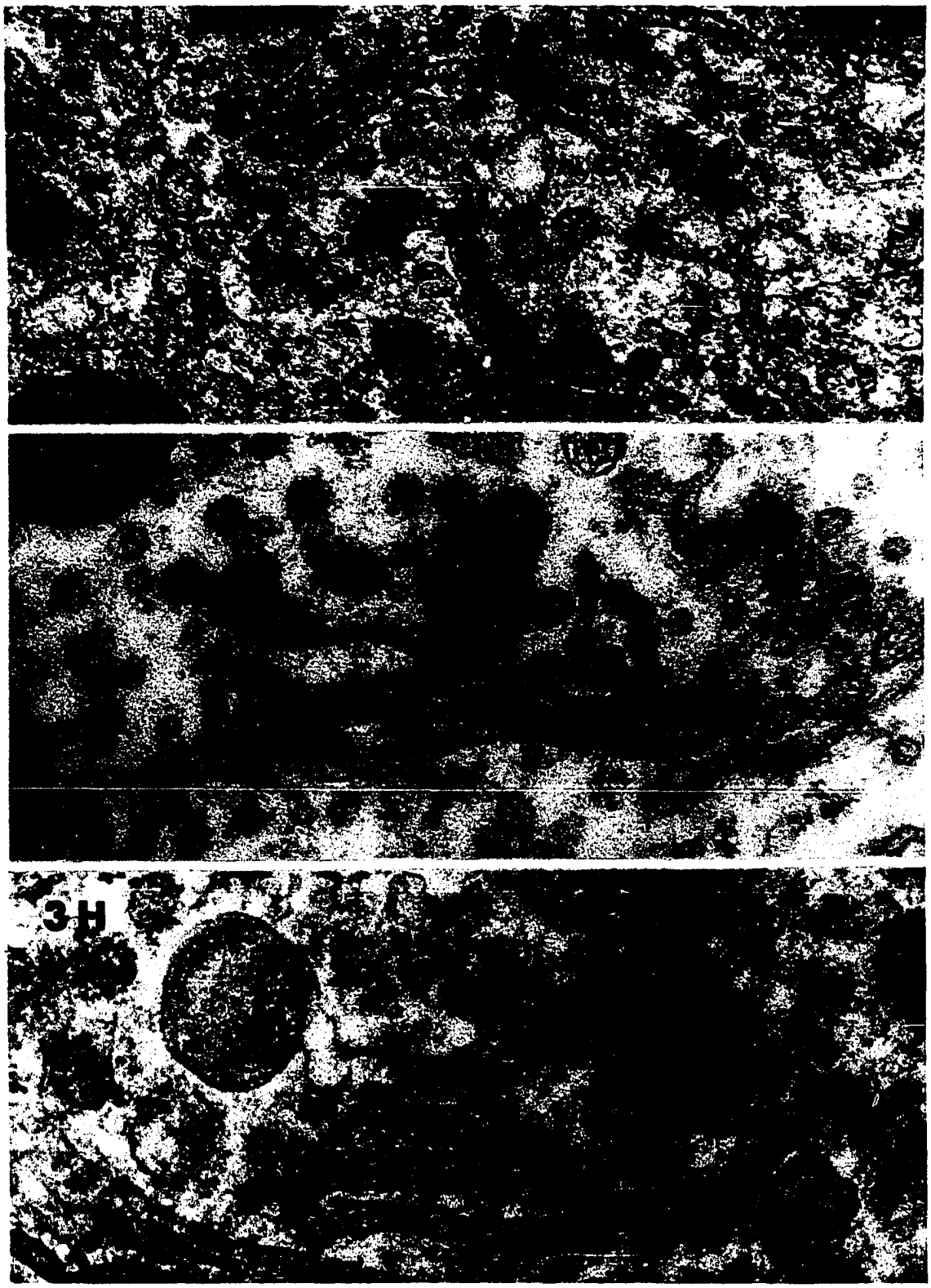
Sugar Nucleotide	Control	Injury	Partial Hepatectomy
UDP-N-acetyl-glucosamine-^{14}C	6417	7018	8010
	9752	9209	----
	7812	12015	6428
	5765	7724	3305
Mean ± s.e.m.	7436 ± 882	8991 ± 1105	5913 ± 1381
Difference (P)	----	N.S.	N.S.
UDP-galactose-^{14}C	17071	19224	16826
	21001	20824	21390
	16825	25020	14596
Mean ± s.e.m.	18299 ± 1352	21688 ± 1728	17604 ± 1998
Difference (P)	----	N.S.	N.S.

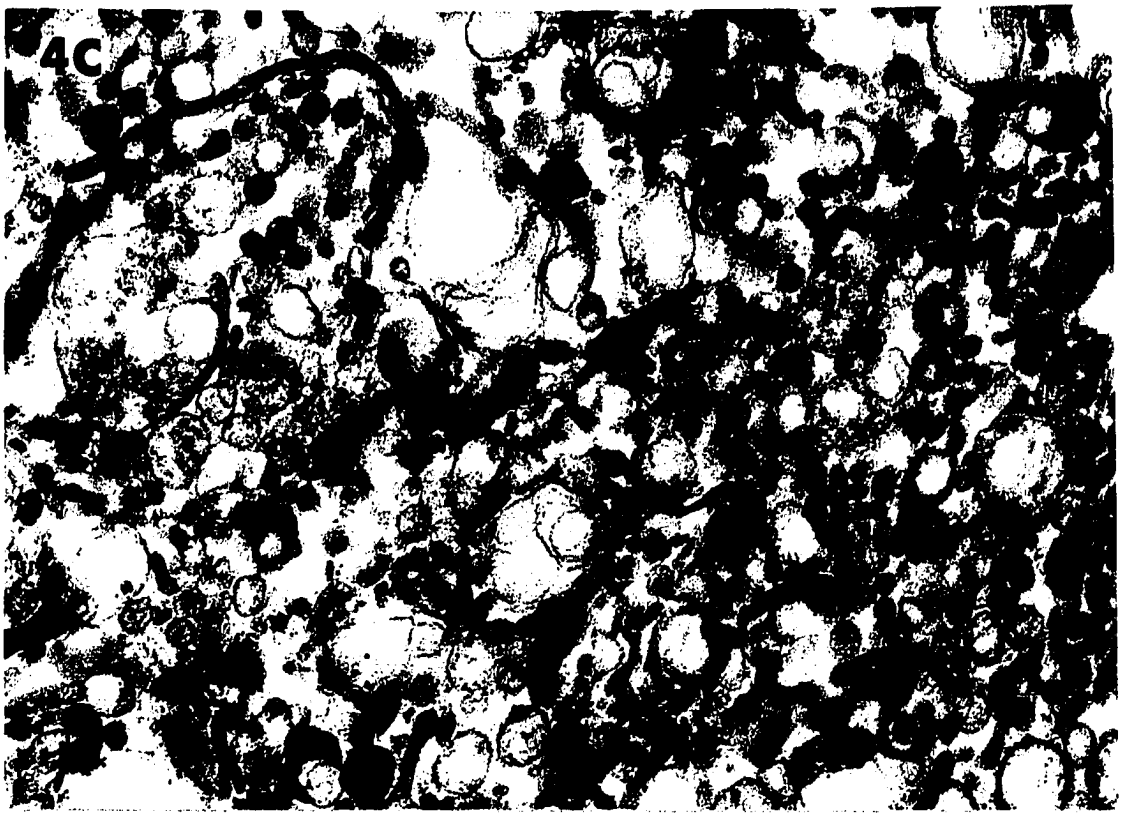
Little or no mannosyltransferase activity was detected in Golgi-rich fractions.

Plates 1-3. Thin sections of portions of liver cells showing intact Golgi apparati at high magnification (x 77,000).

Plates 4-9. Thin sections of isolated Golgi-rich fractions. Plates 4, 6 and 8 present a view at low magnification (x 33,500), and Plates 5, 7 and 9 present a view at high magnification (x 77,000).

Stacks of parallel, secretory vesicles and numerous vesicular profiles can be seen with occasional presence of lysosomes and some electron dense bodies. C, I, and H refer to preparations from control, injured, and partially hepatectomized animals, respectively.


3H


4C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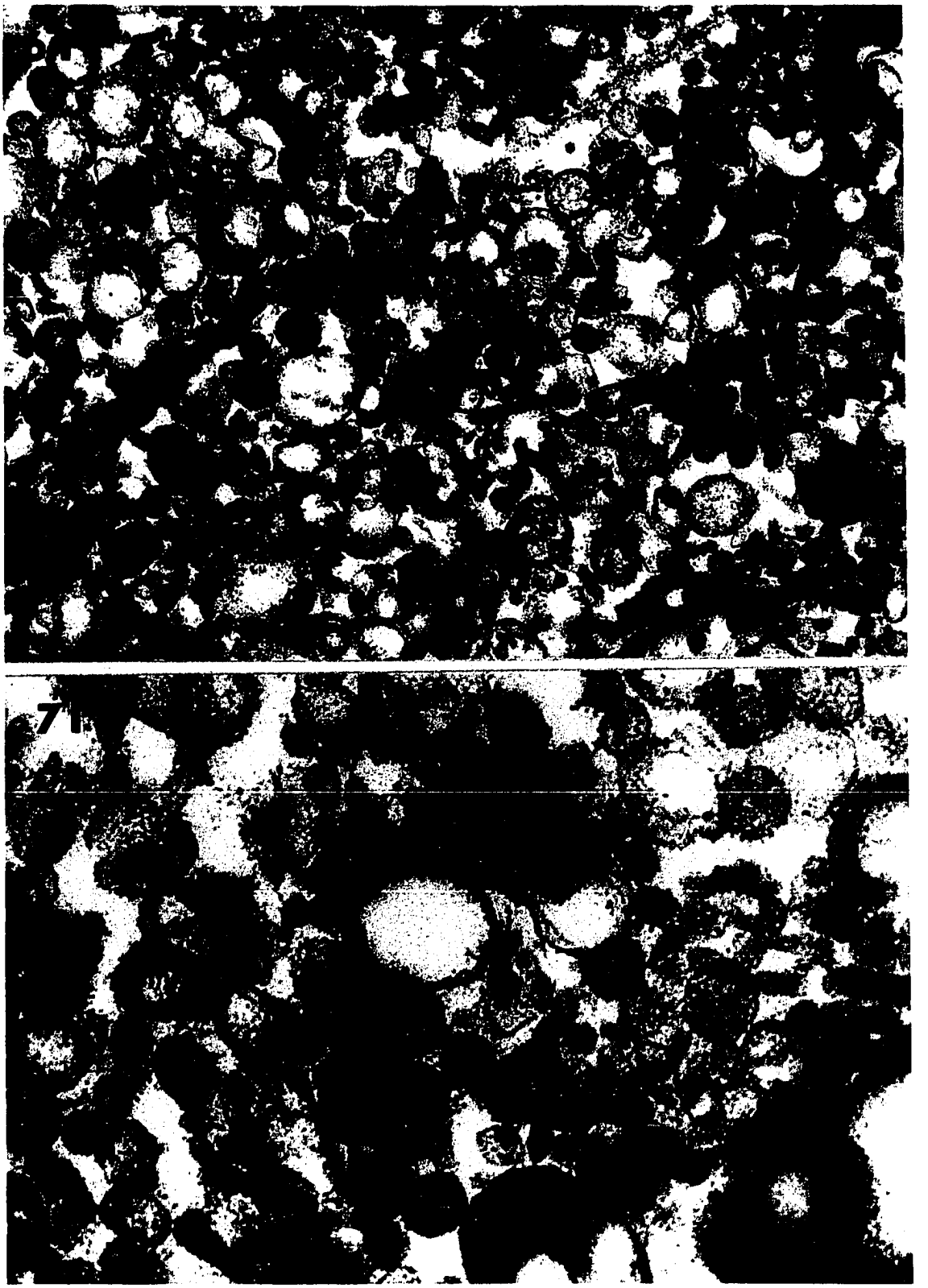

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sacs are sometimes dilated at the ends and contain small secretory vesicles. Occasionally secretory vesicles are found connected to elongated tubules. There are no detectable differences in the morphology of the Golgi bodies of control and experimental liver cells.

Thin sections of Golgi-rich fractions isolated from control, injured and partially hepatectomized rats are shown in Plates 4-9. Typical elements of the Golgi complex are found in abundance. These include cisternae, secretory vesicles and tubular profiles. The electron microscopic assessment indicates that the preparations were 70-80% pure. The main contaminants appeared to be plasma and SER membranes. Occasionally, lysosomes, electron-dense bodies, and mitochondria were detected. As with the whole cells there appeared to be no detectable morphological differences between control and experimental preparations.

Effect of Sugar Nucleotide Supplementation on Monosaccharide Incorporation into Microsomes and Golgi-Rich Fractions

Wagner and Cynkin (226) found that the incorporation of N-acetylglucosamine into Golgi membranes from normal rats was stimulated by the addition of unlabeled GDP-mannose to the reaction mixture. This work was extended to see if similar stimulatory effects on incorporation could be observed with mannose and galactose, as well as with N-acetylglucosamine, into Golgi-rich and microsomal fractions derived from control, injured and partially hepatectomized animals.

The incorporation of monosaccharides was generally stimulated by the presence of other sugar nucleotides when these were added as supplements to the reaction mixture (Table 12). This was true for the three sugars tested in almost all fractions with the exception of

TABLE 12

INCORPORATION OF LABELED MONOSACCHARIDES INTO MICROSOMES AND GOLGI MEMBRANES: EFFECT OF SUGAR NUCLEOTIDE SUPPLEMENTATION

Sugar Nucleotide	Cell Fraction	Control		Injury		Partial hepatectomy	
		dpm/mg protein/hour					
		(-)	(+)	(-)	(+)	(-)	(+)
UDP-N-acetyl-glucosamine-^{14}C ± GDP-mannose / UDP-galactose	Total Microsomes	177	246 (39)*	364	452 (24)	214	321 (50)
	Rough Microsomes	184	178 (--)	200	227 (14)	150	151 (--)
	Smooth Microsomes	363	603 (66)	826	1453 (76)	389	878 (126)
	Golgi Membranes	6338	8897 (40)	9482	12316 (30)	4120	5224 (27)
UDP-galactose-^{14}C ± UDP-N-acetyl-glucosamine / GDP-mannose	Total Microsomes	304	409 (35)	417	756 (81)	293	440 (50)
	Rough Microsomes	166	271 (63)	210	331 (58)	181	385 (113)
	Smooth Microsomes	478	1149 (140)	883	2991 (239)	467	1824 (291)
	Golgi Membranes	37566	59652 (59)	29390	38179 (30)	22662	44912 (98)
GDP-mannose-^{14}C ± UDP-N-acetyl-glucosamine / UDP-galactose	Total Microsomes	207	284 (37)	376	517 (38)	398	599 (51)
	Rough Microsomes	285	458 (61)	491	599 (22)	492	545 (11)
	Smooth Microsomes	178	217 (22)	205	245 (20)	142	228 (61)
	Golgi Membranes†	---	---	---	---	---	---

(-)=Non-supplemented reaction mixture. (+)=Supplemented reaction mixture.
* =Percent stimulation is indicated in parenthesis.
† =Golgi membranes lack mannosyltransferase activity.

N-acetylglucosamine incorporation into the rough microsomal fractions. The stimulatory effect of the sugar nucleotides indicates that the process of heterosaccharide chain elongation in vitro can proceed farther than just one step. The addition of each sugar residue must, therefore, be occurring at the proper position in the sequence and with proper steriochemical specificities, otherwise additional monosaccharide label could not be incorporated. This would also imply that the in vitro incorporation of a monosaccharide is terminated probably because of the saturation of the available acceptor sites. The addition of another sugar gives rise to additional acceptor sites and, consequently, the incorporation of the first monosaccharide can proceed further.

The incorporation of N-acetylglucosamine was stimulated by supplementation with GDP-mannose and UDP-galactose. Since N-acetylglucosamine is largely attached to mannose residues at the growing end of the oligosaccharide chains, this stimulation was probably primarily due to GDP-mannose. The stimulatory activity was concentrated mainly in the smooth microsomal fraction. A similar effect of supplementation with UDP-N-acetylglucosamine and GDP-mannose was seen with galactose incorporation into rough and smooth microsomes, except that here the stimulation was greater than in the case of N-acetylglucosamine incorporation. In this case, probably both of these sugars contribute to the stimulatory effect since both of them occupy a more internal position in the oligosaccharide chain than galactose.

The degree of stimulation of both N-acetylglucosamine and galactose incorporation into microsomes was significantly higher in the case of injury and partial hepatectomy than in the case of controls. A

significant stimulation of N-acetylglucosamine and galactose incorporation was also observed in the Golgi-rich fractions.

The incorporation of mannose into all microsomal fractions was significantly increased by supplementation with UDP-N-acetylglucosamine and UDP-galactose but the stimulation was not as dramatic as in the case of the other two sugars.

Synthesis of Glycolipid Intermediates in the Biosynthesis of Glycoproteins

Several investigators have reported the transfer of sugars from their nucleotide derivatives to lipid acceptors in microsomal membranes (241-244), and they have suggested that these glycolipids may act as intermediates in the biosynthesis of glycoproteins. Since injury and partial hepatectomy stimulate glycoprotein synthesis (155-166), one might expect microsomal glycolipid synthesis to be stimulated as well, particularly if these glycolipids are indeed precursors of glycoproteins. Furthermore, if the syntheses of glycolipids and glycoproteins are closely related to one another as suggested, then it should be possible to show a linear correlation between them following treatments that alter their synthetic rates. In several of the experiments previously described in which labeled monosaccharide transfer to glycoprotein material was measured, an organic-soluble fraction was routinely obtained as part of the work-up of the protein material. This organic-soluble material containing the glycolipids was processed further and counted as well. Several facts emerged from these data. First, all three sugars tested yielded organic-soluble, labeled material, assumed to be glycolipids (241-243). Second, injury and partial hepatectomy increased the amount of label incorporated into the organic-soluble material on a mg

microsomal protein basis. Third, when the glycolipid counts, on a mg microsomal protein basis, were plotted in the form of a scattergram against their corresponding glycoprotein counts, also on a mg microsomal protein basis, good linear correlations were observed for all three sugars (Figures 1 and 2). Glucosamine and mannose incorporation into glycolipids was equivalent to about 10% of the counts incorporated into glycoproteins; for galactose, it was about 6%.

The results obtained here support the idea that glycolipids are intermediates in glycoprotein biosynthesis. It should be pointed out, however, that these results are only suggestive of such a relationship, since it is equally probable that the syntheses of these two kinds of compounds could be simultaneously regulated in the same direction but not directly linked to one another.

Microsomal Glycosyltransferases: Initial Rate Studies

The results of the previous experiments with microsomes clearly indicated that increases in the rate of monosaccharide attachment to endogenous glycoprotein acceptors following injury or partial hepatectomy could be successfully demonstrated in vitro. These experiments, however, did not distinguish between rate increases due to increased endogenous acceptor concentrations and rate increases due to stimulated enzyme activities or both. It has already been shown that stress can stimulate the synthesis of the apoprotein portion of glycoproteins (endogenous acceptors) (155-166), and also that of some sugar nucleotides (249, 269). Both of these act as direct substrates for glycosyltransferases and, therefore, the increases observed in monosaccharide incorporation in experiments based on an incubation time of 60 minutes could be explained

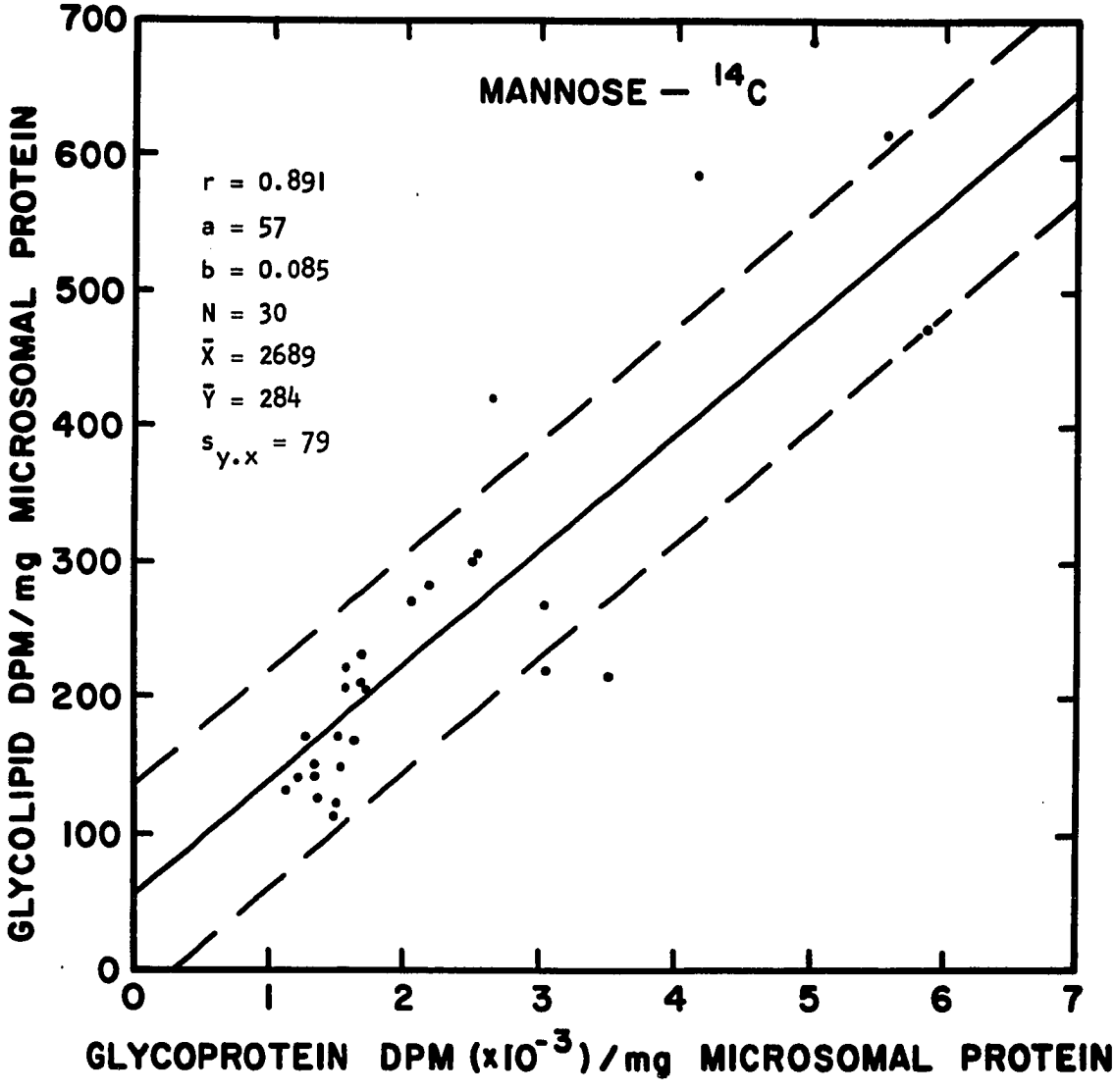

Figure 1. Scattergram showing the correlation between the incorporation of mannose-^{14}C into glycoproteins and glycolipids of microsomal fractions isolated from livers of control, injured and partially hepatectomized rats.

Glycolipids and glycoproteins derived from the same samples of microsomes are plotted against each other.

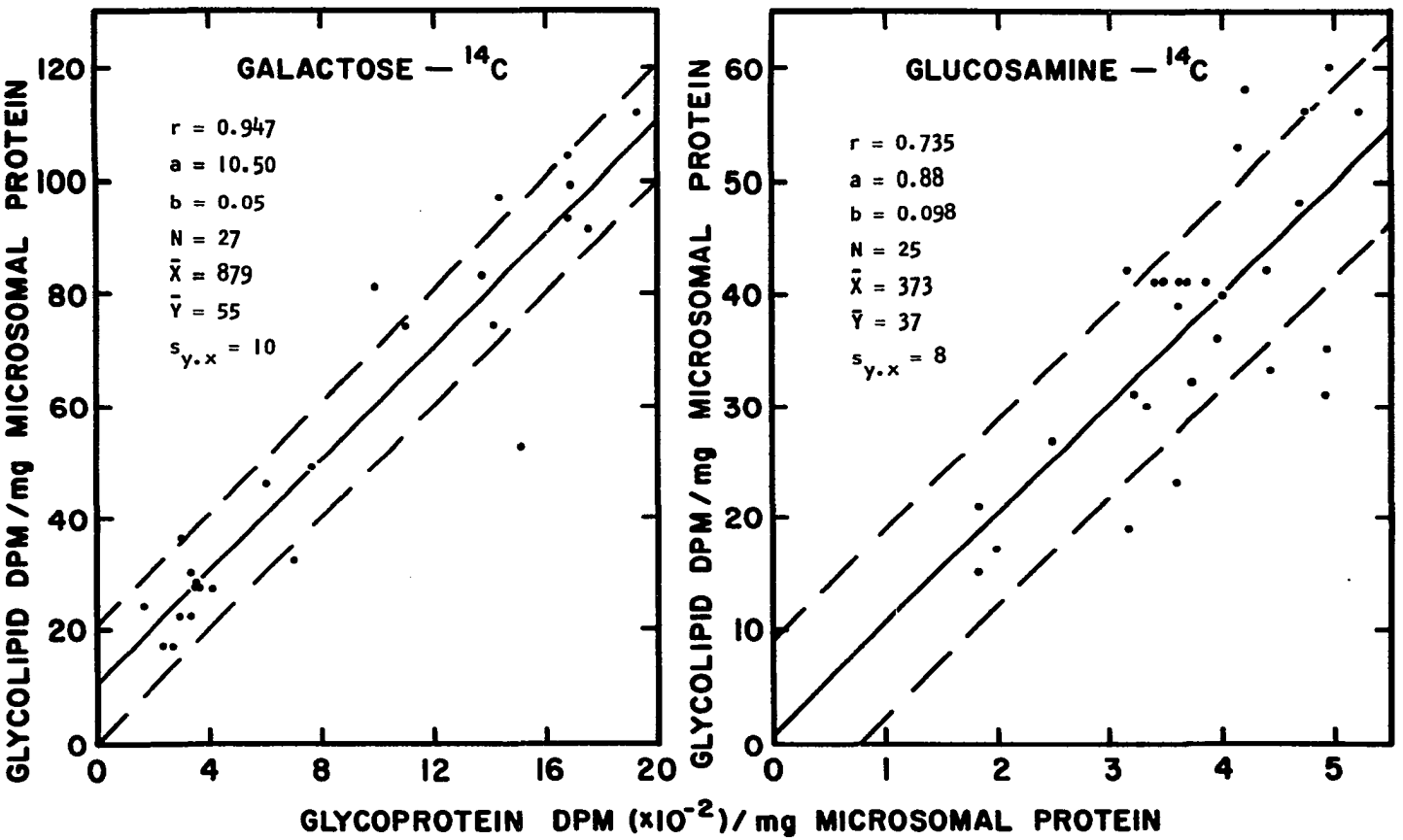

Figure 2. Scattergrams showing correlations between galactose-^{14}C and glucosamine-^{14}C incorporation into glycoproteins and glycolipids of microsomal fractions isolated from livers of control, injured and partially hepatectomized rats.

Glycolipids and glycoproteins derived from the same samples of microsomes are plotted against each other.

on the basis of higher substrate concentrations alone. In the in vitro situation the concentration of sugar nucleotides can be controlled fairly well; thus, we are concerned only with the endogenous acceptor concentrations.

In an attempt to determine if the glycosyltransferases were increased ("induced") as well, a series of experiments were performed in which the initial reaction rates of the glycosyltransferases were measured. The results are presented in Figures 3 and 4.

The initial reaction rates of all three enzymes studied were consistently higher in microsomes isolated from animals subjected to injury or partial hepatectomy when compared to those isolated from controls. In the case of partial hepatectomy, mannosyltransferase activity was even higher than that seen after injury. N-Acetylglucosaminyl- and galactosyltransferase activities, however, were higher only during the initial 2 minutes of the reaction, after which time there was no further incorporation of N-acetylglucosamine or galactose. These results can best be explained by assuming that at this point all the acceptor sites for these two sugars are saturated.

Figure 4 also shows the rate of mannose incorporation into glycolipid. Mannolipid synthesis was markedly stimulated both by injury and partial hepatectomy. The incorporation pattern was similar to that for glycoproteins except that the incorporation into mannolipid was greater after injury than after partial hepatectomy. The inverse was true for incorporation into glycoproteins.

Microsomal Glycosyltransferases: Use of Exogenous Acceptors

The main difficulty encountered in the studies using the

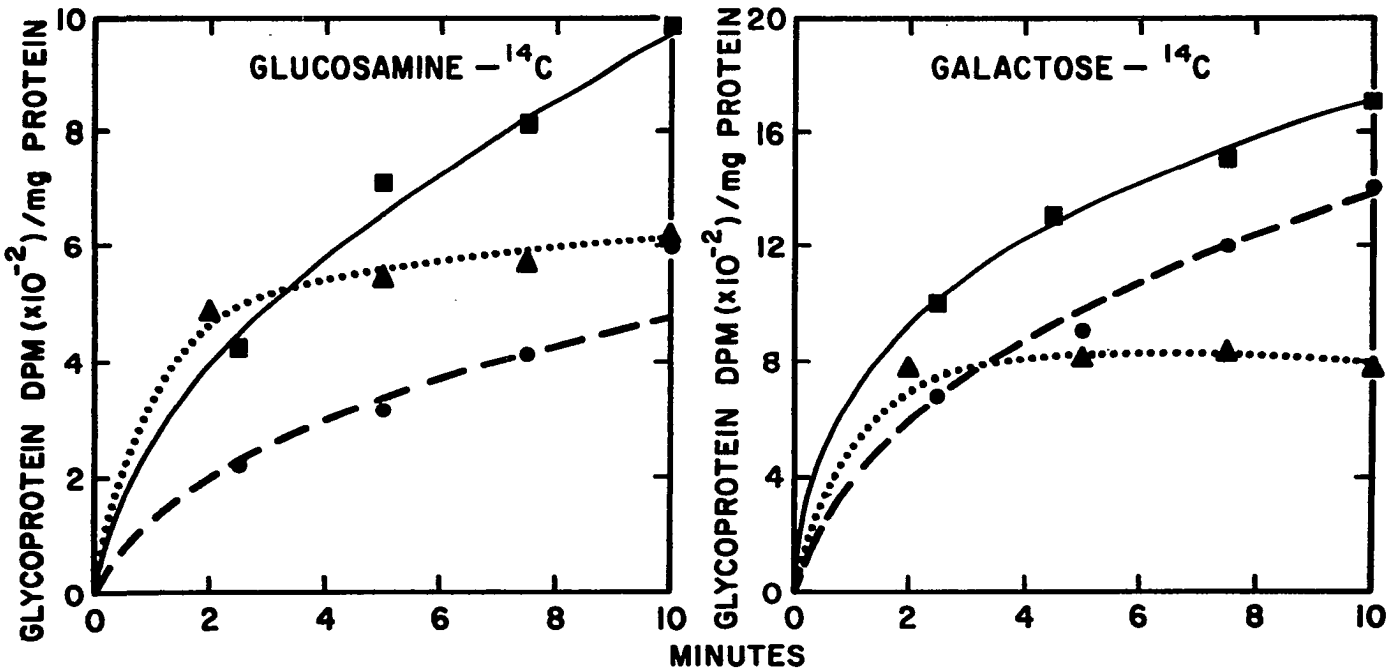

Figure 3. Time course of incorporation of N-acetylglucosamine-^{14}C and galactose-^{14}C into endogenous acceptors of total microsomes.

All assays were carried out in the presence of 1% Triton X-100 and each point represents the results obtained from the pooled liver homogenates of 4 animals.

●—● Control; ■—■ Injury; ▲·····▲ Partial Hepatectomy.

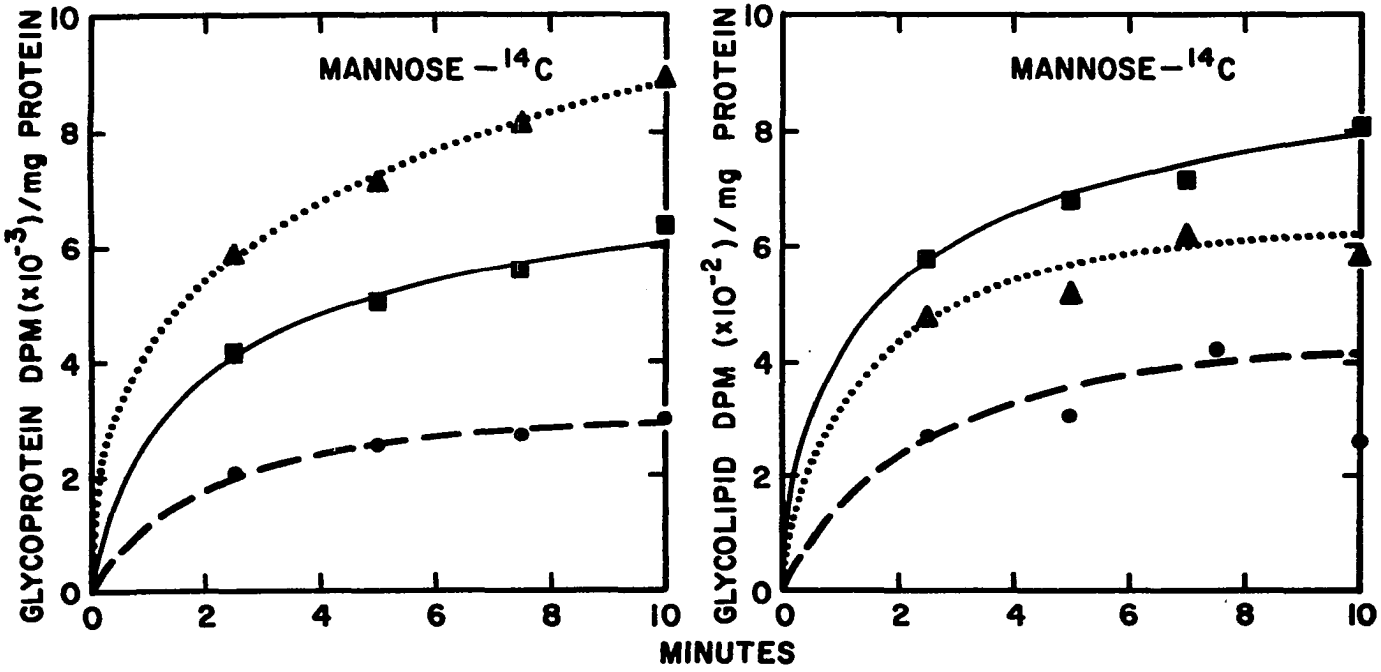

Figure 4. Time course of incorporation of mannose-^{14}C into endogenous acceptors of total microsomes.

All assays were carried out in the presence of 1% Triton X-100 and each point represents the results obtained from the pooled liver homogenates of 4 animals.

●—● Control; ■—■ Injury; ▲····▲ Partial Hepatectomy.

endogenous acceptors was that of the interpretation of the changes observed. Since the concentrations of the endogenous acceptors in these preparations could not be controlled, it was not possible to determine whether the increases in transglycosylation were due to increased enzyme activity or to increased acceptor concentrations or both.

An initial attempt to distinguish between these possibilities was carried out in the "initial rate" studies described above. The results obtained indicated, indirectly, that at least part of the increases occurred as a result of increased enzyme activity.

A more direct assessment of the actual changes in enzyme activities was made by using exogenous acceptors in the assay mixtures. The rationale behind these experiments was straightforward; if the endogenous acceptors are supplemented with a large excess of exogenous acceptors, the acceptor molecules should no longer be the rate-limiting factor in the assay. Any changes observed must, therefore, be the result of actual changes in enzyme activities or levels.

In the experiments described below, two kinds of exogenous acceptors were employed to measure changes in galactosyltransferase activities. The studies were restricted to galactosyltransferase for technical reasons; it is assumed that similar results would have been obtained for glucosaminyltransferase and probably for mannosyltransferase as well.

Lactosamine Synthetase (Galactosyltransferase) Activities in Liver Homogenates

The measurement of lactosamine synthetase activity has been widely used to estimate the purity of Golgi preparations (223-225). This

enzyme, which normally transfers galactose from UDP-galactose to N-acetylglucosamine residues present in the heterosaccharide side-chains of glycoproteins, can also use N-acetylglucosamine per se as a substrate, resulting in the synthesis of N-acetyllactosamine. The synthesis of the latter can be measured and serve as an indicator of galactosyltransferase activity. The results of several such experiments are summarized in Table 13. Each value represents results from liver homogenates pooled from three to four animals.

The activity of galactosyltransferase(s) was consistently and significantly increased (30%) following injury and partial hepatectomy.

Galactosyltransferase Activities of Liver Homogenates Assayed with Exogenous Glycoprotein Acceptors

Once the terminal sialic acid and galactose residues of a glycoprotein are removed it will act as an acceptor in the galactosyltransferase reaction. Two such exogenous acceptors were prepared from orosomucoid and from fetuin. (See Appendix for details for the preparation of these acceptors). Injury resulted in a 20-25% increase in galactosyltransferase activity when compared to controls, and partial hepatectomy resulted in a 38-48% increase (Table 14). Since this experiment was performed one day after injury as well as one day after partial hepatectomy, injury can be used as a control for partial hepatectomy, which gives an increase of 10-20% due to partial hepatectomy alone.

The incorporation of galactose into exogenous acceptors was about 5 times higher than into endogenous acceptors. It is also interesting to note that increases in enzyme activities following injury and partial hepatectomy measured with exogenous acceptors are about one-

TABLE 13

TRANSFER OF GALACTOSE-^{14}C FROM UDP-GALACTOSE-^{14}C TO N-ACETYLGLUCOSAMINE BY ENZYMES PRESENT IN WHOLE LIVER HOMOGENATES

	Control	Injury	Partial Hepatectomy
		dpm/mg protein/5 min.	
	1134	2640	3000
	1312	1345	1887
	1980	2602	2246
	2114	2838	2947
	4000	5251	4303
	10885	13151	12731
Mean ± s.e.m.	3571 ± 1526	4638 ± 1787	4519 ± 1677
Difference (P)	----	0.025	<0.025

Assays were carried out as described in Methods. The values presented in this table represent only the amount of galactose-^{14}C present in N-acetyllactosamine-^{14}C and has been corrected for the release of galactose-^{14}C resulting from nonspecific hydrolysis of UDP-galactose-^{14}C. Galactose-^{14}C transferred to the endogenous acceptors was excluded by passing the reaction mixture through Dowex columns.

TABLE 14

TRANSFER OF GALACTOSE-^{14}C FROM UDP-GALACTOSE-^{14}C TO EXOGENOUS ACCEPTORS BY ENZYMES PRESENT IN WHOLE LIVER HOMOGENATES

Acceptor	Control	Injury	Partial Hepatectomy
	dpm/mg acceptor/mg liver protein/hour		
Endogenous Glycoproteins			
Mean ± s.e.m.	688 ± 31	1075 ± 22	1346 ± 150
No. of Observations	5	5	5
Difference (P)	----	<0.001	<0.001
Fetuin			
Mean ± s.e.m.	4348 ± 148	5424 ± 217	6019 ± 642
No. of Observations	5	5	5
Difference (P)	----	<0.005	<0.025
Orosomucoid			
Mean ± s.e.m.	4060 ± 185	4941 ± 110	6036 ± 572
No. of Observations	5	5	5
Difference (P)	----	<0.001	<0.025

Liver homogenates were diluted with 9 volumes of 1% Triton X-100 to solubilize enzymes and acceptors; 0.01 ml was used in the assay. Values for radioactivity incorporated into exogenous acceptors were obtained by subtracting the radioactivity incorporated into endogenous acceptors from the total radioactivity incorporated into exogenous and endogenous acceptors. Assays were carried out essentially as described in Methods for the "lactosamine synthetase assay," except that 3 mg of exogenous acceptors were used in each assay tube instead of N-acetyl-glucosamine and the reaction was stopped with 5% phosphotungstic acid solution in 2 N HCl one hour after incubation.

half of those measured with endogenous glycoprotein acceptors. The increases in endogenous acceptors could be responsible for about one-half the total increases observed in monosaccharide transfer; the other half may be taken as representative of increases in actual enzyme activities or levels.

Time of Maximum Response Following Surgery

The stimulation of plasma glycoprotein synthesis following injury and partial hepatectomy shows a characteristic time-response pattern. If the transferase increases observed in these studies are closely linked to plasma glycoprotein biosynthesis then one might expect a similar or identical time-response pattern to be exhibited by these enzymes as well. A preliminary experiment was carried out using mannosyltransferase as a representative enzyme. The results are shown in Figure 5. The time of maximal mannosyltransferase activity was approximately 18 hours following surgery, and is in agreement with maxima reported previously for cell-free amino acid incorporation and polysomal aggregation (160), seromucoid synthesis (156), and glucosamine synthetase activity (249). Although an 18 hour peak was also observed after partial hepatectomy, a large part of this could have been due to a general stress response. If injury is used as a control for partial hepatectomy, the incorporation of mannose into microsomes of regenerating rat livers appears to reach a maximum between 24 and 36 hours after surgery, which is the time of maximum cell proliferation (270).

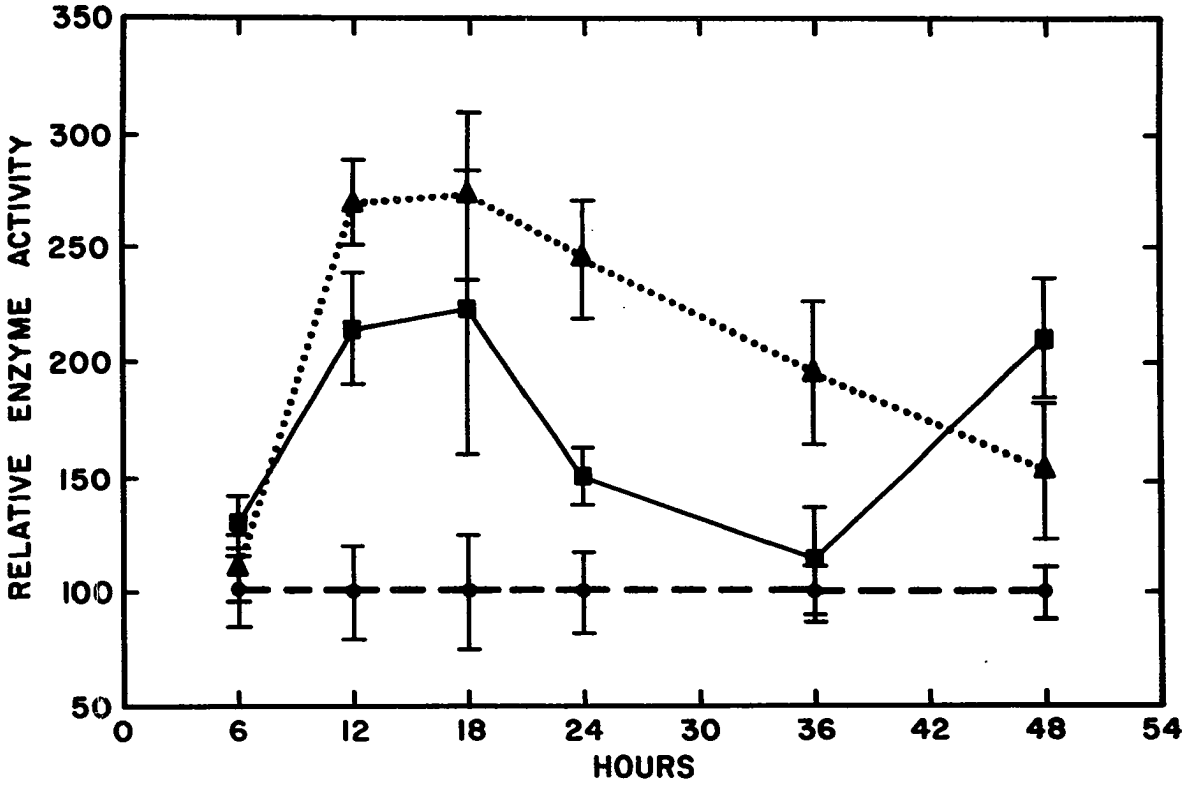

Figure 5. Mannosyltransferase activity as a function of time following Injury ■—■, and Partial Hepatectomy ▲····▲ expressed as percent of Control ●— -●.

Values are presented as means ± s.e.m. of 4 separate observations.

CHAPTER IV

DISCUSSION

Biosynthesis of Glycoproteins on Polyribosomes: Effect of Injury and Partial Hepatectomy

Many reports have been published recently supporting the concept that some polyribosomes are bound to membranes of the endoplasmic reticulum whereas others are found free in the cytoplasm of eukaryotic cells (271-275). The functional differences between the two kinds of polyribosomes in synthesizing secretory and sedentary proteins seem to be well documented (143-149). Membrane-bound ribosomes are not only found in fully differentiated tissues, such as liver or pancreas which synthesize and secrete large amounts of proteins, but also in cells like HeLa cells, which have no apparent secretory function (276). It has been suggested that bound polysomes also synthesize membrane proteins in addition to secretory proteins and this might explain their presence in cells that do not secrete macromolecules (277).

The stress-induced increase in the amount of membrane-bound polysomes in the rat liver is another demonstration of functional differences between free and bound polysomes. The present work supports previous findings (152-154, 174) that glycoproteins are primarily synthesized on membrane-bound polysomes. Since the ratio of membrane-bound polysomes to free polysomes doubles after injury, one might expect to see an

increase in the RER. In the regenerating liver, on the other hand, the amount of free polysomes is greatly increased and the bound to free polysome ratio actually decreases. One way of explaining this observation would be to assume that in a dividing cell mass there is a greater need for the synthesis of non-exportable, intracellular proteins than for secretory proteins. If membrane proteins are also synthesized on membrane-bound polysomes, one might also expect to see an increase in bound polysomes in the regenerating liver, since membrane synthesis is enhanced to meet the growing needs of the dividing cells. The present work does not seem to support this idea.

Recent reports (271, 272) that steroid hormones may be involved in promoting the binding of ribosomes to the membranes are of particular interest in connection with the stress-induced increases in membrane-bound polysomes. Since stress is known to elicit a greater secretion of adrenocortical hormones (74), corticosteroids may play an important role in the increased binding of ribosomes to the membranes. A study devoted to the effects of adrenalectomy, castration and hypophysectomy on the stress-induced response of glycoprotein synthesis certainly deserves attention. Since corticosterone has been found to enhance binding and hydrocortisone to inhibit binding of ribosomes to rat liver membranes treated with EDTA (271), the ratio of these two hormones might be important for ribosome-membrane interactions and could explain some of the controversies regarding the action of corticosteroids on glycoprotein synthesis.

Since most of the monosomes and disomes were supposedly eliminated during the isolation of polyribosomes by the method of Blobel

and Potter (141), it is not surprising that the polysomal preparations from control and experimental animals were found to be equally active in amino acid incorporating systems and had the same RNA/protein ratios. Although the amount of polysomes is increased after stress, their specific activities do not seem to be changed significantly. Thus it is the quantity and not the quality of polysomes that is changed by stress.

Effect of Injury and Partial Hepatectomy on Monosaccharide Incorporation into Subcellular Fractions

Injury and partial hepatectomy were generally followed by an increase in monosaccharide incorporation into the various subcellular fractions examined in these experiments. Since measured, uniform amounts of sugar nucleotides were used in the *in vitro* systems, any increases in monosaccharide transfer have to be ascribed to increases in either the concentration of endogenous glycoprotein acceptors, or in the activities of the transferases, or possibly both. Since the microsomal incorporation of all three sugars increased to the same extent (40%) after injury, it may be assumed that all three sugars were added to the same acceptors. Microsomes from the regenerating livers, however, did not show any significant increases in N-acetylglucosamine or galactose incorporation, although mannose incorporation was higher than that seen after injury. There could be two reasons why microsomes from the regenerating liver did not show a higher activity in N-acetylglucosamine and galactose incorporation: (i) The concentration of endogenous acceptors was not increased after partial hepatectomy, or (ii) a part of these endogenous acceptors was unable to accept N-acetylglucosamine or galactose residues. The first possibility can be ruled out on the basis of the following

arguments: (a) An increase in the rate of plasma glycoprotein biosynthesis normally seen after partial hepatectomy, could not occur without an increase in acceptor molecules; (b) Since all three sugars are presumably added to the same endogenous acceptors, it seems improbable that the concentration of the mannose acceptors was increased after partial hepatectomy without changing the concentrations of N-acetylglucosamine and galactose acceptors. Therefore, the second possibility that a part of the acceptors was not yet ready to accept the latter two sugars seems to be more reasonable.

When one carefully examines the sugar sequences of the "inner core" of glycoproteins one finds that the innermost N-acetylglucosamine residue is usually followed by mannose and then by N-acetylglucosamine and galactose:

-Asn-(GlcNAc)-(Man)-(GlcNAc)-(Gal)-(NANA)

If it is assumed that the innermost N-acetylglucosamine residues are largely attached to the nascent polypeptide chains <u>in vivo</u>, the results obtained above can be explained as follows: whereas the incorporation of all three sugars (mannose, N-acetylglucosamine, and galactose) into microsomes is increased following injury, the <u>in vivo</u> incorporation of mannose cannot keep pace with the increased synthesis of endogenous acceptors after partial hepatectomy. Consequently, some of these acceptor molecules lack mannose and, therefore, cannot act as acceptors for subsequent N-acetylglucosamine and galactose residues. In other words, these acceptors are "mannose-deficient."

The above explanation is further supported by results obtained in the experiments with sugar nucleotide supplementation. When unlabeled

GDP-mannose was included in the reaction mixture, the incorporation of N-acetylglucosamine and galactose was stimulated to a greater extent into microsomes from the regenerating liver than into equivalent preparations from normal controls or injured animals. It is, therefore, clear that the endogenous acceptors as well as the transferase(s) for mannose are present in higher concentrations in microsomes from the regenerating liver than in microsomes from control livers. This leaves GDP-mannose, or another early mannose intermediate, as a limiting substrate in the regenerating liver, which, when added from outside, causes marked stimulation of mannose incorporation into microsomes. The results presented in Figure 4 are also consistent with the idea that the regenerating liver exhibits a mannose transfer deficiency. Thus the supply of mannose could be a natural method of regulation of glycoprotein biosynthesis under certain conditions.

If the mannose deficiency concept proves to be true, it could also provide a reasonable explanation for a relatively poor incorporation of N-acetylglucosamine into postmicrosomal particles isolated from the regenerating liver.

The study of glycoprotein side-chain microheterogeneity has not yet been extended to glycoproteins synthesized by stressed or by partially hepatectomized animals, but it would be interesting to see if there is an increase in heterogeneity under these conditions, expecially if there is a deficiency of mannose in the regenerating liver. Experiments by Ali and Chandler (278) using concanavalin A as an investigative tool to observe changes in glycoprotein oligosaccharides (microheterogeneities) gave some support to the idea that plasma proteins synthesized by

regenerating livers have altered side-chains.

The extension of the sugar incorporation studies to microsomal subfractions showed that the mannosyltransferase activities were localized primarily in the rough microsomal fraction, while the galactosyltransferase activities were found mainly in the smooth membrane fractions. This is in good agreement with results reported by others (209-212). Glucosaminyltransferase activities, however, were found to be higher in smooth microsomes than rough microsomes. These results agree with those reported by several investigators (209, 212) but contradict those reported by Molnar and coworkers (216). Our results are compatible with the idea that the innermost N-acetylglucosamine residues are largely attached to the nascent polypeptides *in vivo* and that the incorporation observed *in vitro* represents primarily the addition of those N-acetylglucosamine residues which are peripheral to mannose. This is also supported by studies on the *in vivo* incorporation of glucosamine into smooth microsomes, which were twice as active in glucosamine uptake as were rough microsomes. The discrepancies in results obtained by various investigators may be due to differences in the preparation of rough and smooth microsomes. Since Molnar and coworkers (216) have not reported on the RNA or phospholipid content of their preparations, it is not possible to compare our preparations with theirs. A comparison of the rough *versus* the smooth microsomal fractions on the basis of the specific activities of monosaccharide incorporation alone may, however, be deceptive since the rough microsomes contain large amounts of ribosomal proteins which will tend to lower their specific activities.

The increases observed in mannose incorporation into the total

microsomes after partial hepatectomy can be traced to the rough microsomal fraction and those in N-acetylglucosamine and galactose incorporation following injury can be traced to the smooth microsomes. Since stimulation of N-acetylglucosamine and galactose incorporation by GDP-mannose was much greater in smooth microsomes than in rough microsomes, one might suppose that glycopeptides were being passed on from the rough to the smooth microsomes prematurely, lacking an adequate number of mannose residues.

The Golgi-rich fractions isolated from control and experimental animals did not differ in in vitro monosaccharide incorporating abilities (Table 11); i.e., they were equally active on a mg protein basis. Thus, in order to achieve an overall increase in glycoprotein synthesis, the transport of these macromolecules through the Golgi membranes must be enhanced, provided all glycoproteins are processed and secreted via this organelle. This can be accomplished by (a) increasing the amount of Golgi membranes and, therefore, the number of multiglycosyltransferase units per cell, (b) increasing the rate of flow of glycoproteins through these membranes, or (c) both.

If one accepts the conclusions of Roseman and coworkers (168) and Schachter and colleagues (279) that galactosyl- and sialytransferases are specifically confined to the Golgi membranes, then the above results can be explained on the basis of an increase in these membranes following injury and partial hepatectomy, since the total galactosyltransferase activity in whole liver homogenates was increased 25-35% under these conditions (Table 14). The phospholipid to DNA ratios (Table 10), which were supposed to represent the amount of membranes per cell, did not,

however, provide any evidence for such an increase.

It is possible that the amount of Golgi membranes remained constant but injury and partial hepatectomy stimulated the rates of transport of glycoproteins through these membranes. If the membranes have a limited capacity to bind acceptors, then it is possible that the in vitro assays would fail to detect these rate increases. Our results showed that endogenous acceptors "piled up" in the RER and SER after injury and partial hepatectomy, indicating that the Golgi processing might be rate-limiting. Thus, if the activity of the Golgi membranes to process glycoproteins was increased, this increase was not enough to prevent the "piling-up" and was not detectable on the specific activity basis in the in vitro assays.

Glycolipids as Intermediates in the Biosynthesis of Glycoproteins

The linear correlations observed between N-acetylglucosamine, galactose, and mannose incorporation into glycoproteins and glycolipids strongly suggests that the biosyntheses of these two classes of macromolecules are closely related. Since the linear correlations between the two were not affected by injury or partial hepatectomy, it may be concluded that glycolipid synthesis increased concomitantly with an increase in glycoprotein synthesis. The incorporation of mannose, in particular, into glycolipids and glycoproteins (Figure 4) certainly supports this view. These results are consistent with and support the earlier suggestions (241-245) that glycolipids may serve as intermediates in the biosynthesis of glycoproteins. Although glucosamine and galactose are constituents of many other glycolipids, such as ceramides and cerebrosides, mannose is not found as a component of these glycolipids (280).

It seems probable, therefore, that these glycolipids represent lipid intermediates in the biosynthesis of glycoproteins and are not glycolipids of the kind of ceramides and cerebrosides. Although the results presented in this dissertation seem to fit nicely into the "lipid intermediate hypothesis," other possible explanations should not be ignored. The linear relationships observed between glycolipids and glycoproteins suggest only that their syntheses may be regulated together. It is quite possible, however, that the syntheses of the two are increased independently and that one may not necessarily be a precursor to the other. The "lipid intermediate hypothesis" is still at an early stage of development and open to many questions. For instance, it is not clear whether the synthesis of glycolipids involves the same transferases used for glycoprotein synthesis or different enzymes. What is the precise nature of these intermediates and how are they synthesized? Since purified glycosyltransferases are capable of transferring monosaccharides directly from sugar nucleotides to purified glycoprotein acceptors in vitro (231), one wonders if the synthesis of glycolipids indeed precedes glycoprotein synthesis in vivo. A definite proof that glycolipids serve as intermediates in the biosynthesis of glycoproteins certainly requires identification of these intermediates, their chemical synthesis, and demonstration in vivo and in vitro that they indeed are intermediates in the biosynthesis of glycoproteins.

Effect of Injury and Partial Hepatectomy on the Activities of Glycosyltransferases

The initial rate studies were the first in this series to indicate that the activities of glycosyltransferases were increased

following injury and partial hepatectomy. Since the initial rates of transfer of all three sugars studied were found to be consistently higher in microsomes isolated from injured animals when compared to those from normal controls, it was tentatively concluded that the activities of all three microsomal transferases were increased after stress. In the case of partial hepatectomy, the rate of mannose transfer was found to be even higher than in the case of injury.

The rates of N-acetylglucosamine and galactose transfer, however, were higher than the control values only up to 2 minutes incubation time, after which time they leveled off. This could either mean that the enzymes were inactivated or that the acceptor sites were fully saturated at this point. The inactivation of enzymes seems unlikely since supplementation with GDP-mannose was effective in stimulating N-acetylglucosamine and galactose transfer to endogenous proteins. Moreover, galactose transfer was greater after partial hepatectomy than in controls when measured with exogenous acceptors. Therefore, these results can be explained by assuming that the supply of endogenous acceptor sites for N-acetylglucosamine and galactose were exhausted after a 2-minute incubation of microsomes isolated from the regenerating liver. The initial rate experiments performed with endogenous acceptors were not definitive, however, but only suggestive of increases in enzyme activities and should be repeated using exogenous acceptors.

The use of exogenous glycoprotein acceptors in the transferase assay mixtures clearly demonstrated that there was a significant increase in the activities of galactosyltransferases following injury and partial hepatectomy. The increases in galactosyltransferase activities measured

by three different exogenous acceptors (N-acetylglucosamine, and the fetuin and orosomucoid derivatives) were remarkably similar (about 30%). This is in good agreement with the previous reports (168, 232) that galactose is transferred to N-acetylglucosamine by the same enzyme that transfers this sugar to the glycoprotein acceptors.

The activities of N-acetylglucosaminyl- and mannosyltransferases could not be assayed with exogenous acceptors because of the non-availability of sufficiently pure N-acetylglucosaminidase and mannosidase to prepare them, but it appears from the initial rate studies that their activities were also increased following stress. In fact, if the incorporation of galactose and that of N-acetylglucosamine into the same samples of liver microsomes isolated from control, injured and partially hepatectomized animals are plotted against each other on a scattergram, a good linear correlation is observed between the two (Figure 6). Since the linear relationship between N-acetylglucosaminyl- and galactosyltransferase activities was not disturbed after injury or partial hepatectomy, there is a good possibility that the activities of both these enzymes were increased in parallel under these conditions. Similarly, mannosyltransferase activities were probably also increased following injury and partial hepatectomy, but confirmation of this requires the use of exogenous substrates.

Hudgin et al. (279) recently reported increases in sialyltransferase activities in the livers of rats bearing Morris hepatomas 7777, 7800, and 5123D. They were also able to see a significant increase in N-acetylglucosaminyltransferase activity in the livers of animals carrying hepatoma 7777, but not in those carrying hepatomas 7800 and

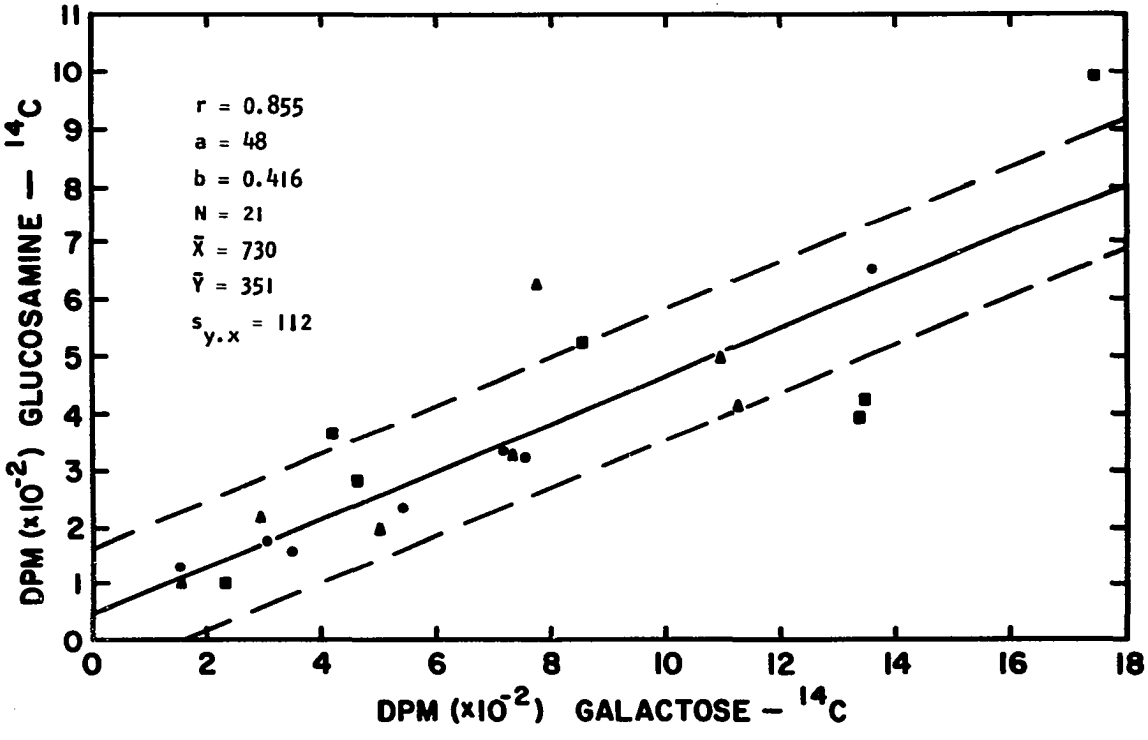

Figure 6. Scattergram showing the correlation between galactose-^{14}C and N-acetylglucosamine-^{14}C incorporation into microsomal fractions isolated from Control ● ● ●, Injured ■ ■ ■ and Partially Hepatectomized ▲ ▲ ▲ rats.

5123D. Results obtained in the present study are in good accord with the above reports and together suggest that the activities of most, if not all, transferases are increased after stress. These findings also support the thesis that the mechanisms responsible for producing the stress-specific response are remarkably similar no matter whether the stress stimulus is applied by physical or pathological means.

In the light of the present and previously reported work, it seems logical to propose that stress stimulates the synthesis of both the protein as well as the carbohydrate moieties of glycoproteins simultaneously. Since the regulatory control is quite specific in that the concentrations of only a selected number of glycoproteins increase after stress, one will have to assume that this specificity is conferred primarily by a control on the synthesis of the protein moiety. An increase in the activities of glycosyltransferases may be considered obligative to increases in the concentrations of apoprotein acceptors and some carbohydrate precursors. There is, however, one way that these transferases can also exert a specific effect on glycoprotein synthesis. If it is assumed that the carbohydrate moiety of each glycoprotein is synthesized by a separate set of transferases, then the activities of only a few specific "sets of glycosyltransferases" might be increased after stress. There is no clear-cut evidence as yet that the synthesis of the carbohydrate moiety of each glycoprotein requires separate and specific transferases. Bosmann's findings that the activity of N-acetylglucosaminyltransferase purified from guinea-pig liver was much higher when measured with fetuin acceptors than that measured with orosomucoid acceptors (231) raises the possibility that the transferases might be protein-specific.

The results of the present study do not seem to support this idea. The fact that rat liver enzyme can use glycoprotein acceptors prepared from human or calf serum proteins as substrates quite efficiently, negates the idea of such a high specificity. In fact, glycoprotein acceptors and N-acetylglucosamine compete for the same sites on galactosyltransferase (232).

It is interesting to note that the time of maximal activity of glycosyltransferases following stress coincides with the time when the synthesis of the apoprotein part of glycoproteins is maximal. This further supports the view that the syntheses of the carbohydrate and protein moieties of glycoproteins are regulated simultaneously. In view of the complexity of extending regulatory control over a number of synthetic processes in response to stress, it appears unlikely, although not impossible, that only one hormone or factor is responsible for bringing about all these changes. It may be more reasonable to think that these changes are a result of a collective effort of several factors or hormones released after stress. What these hormones are remains to be clarified. The area of hormonal regulation of glycoprotein synthesis certainly needs to be explored further using new techniques and more direct approaches.

CHAPTER V

SUMMARY

Regulation of glycoprotein biosynthesis in the rat liver was studied using injury and partial hepatectomy as experimental models. This work included studies on the functional differentiation between free and membrane-bound polysomes, and the in vitro transfer of monosaccharides to endogenous and exogenous glycoprotein acceptors by enzymes present in the microsomal and Golgi-rich fractions.

Marked alterations in the relative distribution of membrane-free and membrane-bound polysomes were detected following injury and partial hepatectomy. Injury caused a two-fold increase in the membrane-bound to free polysome ratio, primarily due to an increase in membrane-bound polysomes. Partial hepatectomy, on the other hand, resulted in a decrease in membrane-bound to free polysome ratio, mainly because of an increase in free polysomes. These observations were consistent with the suggested functional segregation of the two polysomal populations, that is, the exportable secretory porteins are synthesized on membrane-bound polysomes, while the non-exportable intracellular proteins are synthesized on free polysomes.

Purified, detergent-treated, polysomes from control and experimental animals showed comparable activities in cell-free amino acid

incorporation systems and injury or partial hepatectomy caused no significant changes in the RNA to protein ratios of these preparations.

The *in vitro* incorporation of N-acetylglucosamine, galactose and mannose into glycoproteins and glycolipids of microsomal fractions was significantly higher after injury or partial hepatectomy than in normal controls. The endogenous acceptors present in microsomes isolated from regenerating livers appeared to be deficient in mannose, suggesting a limitation in the supply of some mannose intermediate(s) after partial hepatectomy. The regulation of the mannose supply may be a natural means of regulation of glycoprotein biosynthesis.

Golgi-rich fractions isolated from control, injured and partially hepatectomized animals showed no significant differences in the rate of N-acetylglucosamine or galactose incorporation *in vitro*. Similarly, no significant differences could be detected in the morphology of the three preparations by electron microscopic examination.

A linear correlation between the incorporation of monosaccharides into glycoproteins and glycolipids was established for all three sugars studied, which is consistent with the possibility that glycolipids may be intermediates in the biosynthesis of glycoproteins.

Initial rate studies indicated that the increases in the rates of monosaccharide transfer to endogenous glycoprotein acceptors following injury and partial hepatectomy were, in part, due to increases in the activities of the glycosyltransferases. Galactosyltransferase activities assayed using fetuin and orosomucoid derivatives as exogenous acceptors showed a 30-40% increase in the enzyme activity in whole liver homogenates, following injury and partial hepatectomy. The same results were

obtained when N-acetylglucosamine was used as an exogenous acceptor. The activities of N-acetylglucosaminyl- and mannosyltransferases were not measured by using exogenous acceptors, but a linear correlation was observed between galactose and N-acetylglucosamine transfer to endogenous acceptors of liver microsomes isolated from control, injured and partially hepatectomized animals. It is suggested that the activities of all three transferases are increased under these conditions.

The in vitro transfer of mannose measured as a function of time after stress reached a peak at 18 hours, a value in excellent agreement with that reported for maximal incorporation of amino acids into microsomes, maximal polysomal aggregation, maximal glucosamine synthesis and overall increase in plasma glycoprotein biosynthesis.

These results are discussed in relation to the overall coordinated synthesis of the carbohydrate and apoprotein moieties of glycoproteins.

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APPENDIX

Isolation of Fetuin

Fetuin was isolated by the method of Spiro (281). Two volumes of 0.03 M zinc acetate solution in 28.5% ethanol were added dropwise to 300 ml of fetal calf serum at -5° with constant stirring. The mixture was adjusted to pH 6.4 with 1 M NH_4OH-NH_4Cl buffer, pH 10.4, in 19% ethanol and was allowed to stand for 15 hours at -5°. This step precipitated hemoglobin and most of the plasma proteins other than fetuin. The precipitate was removed by centrifugation and discarded. Enough 1 M barium acetate at -5° was added to the supernatant to give a final concentration of 0.02 M with respect to barium. Ethanol was prechilled to -70° and was added dropwise to the mixture to obtain a final concentration of 25%. The mixture was adjusted to pH 6.7 and allowed to stand for 2 hours. A slight precipitate appeared which was removed by centrifugation and discarded. Fetuin was precipitated from this supernatant by the dropwise addition of 95% ethanol (-70°) at -10° to give a final ethanol concentration of 40%. After standing for 12 hours, the fetuin precipitate was recovered by centrifugation, dissolved in 50 ml of 0.1 M trisodium citrate, and dialyzed for 3 days against 4 changes of 4 liters of distilled water. The dialyzed solution was passed through a mixed-bed ion-exchange resin to remove traces of heavy metals and was lyophilized and stored at -20°.

Isolation of Orosomucoid

Orosomucoid was isolated from human plasma as described by Michon et al. (282). To a measured volume of plasma, enough ammonium sulfate was added dropwise from a 4 M solution, pH 7.0, at 0°, to obtain a final ammonium sulfate concentration of 2.4 M. After standing for 12-16 hours at 0-4°, the precipitate was removed by centrifugation, washed with 2.4 M ammonium sulfate solution, pH 7.0, and the washings were added to the previous supernatant. The combined supernatant was adjusted to pH 5.0 with 1 N H_2SO_4 and allowed to stand for 2 hours. The precipitate was removed by centrifugation and washed with 2.4 M ammonium sulfate, pH 5.0. The precipitate was discarded and the washings were added to the supernatant obtained above. Solid ammonium sulfate was added to the combined supernatants to bring the solution to saturation, after which the pH dropped to 4.0. The mixture was allowed to stand for 24 hours at 0-4°. The precipitate formed was collected, dissolved in water and dialyzed against running tap water and then distilled water until all ammonium sulfate was removed. The dialyzed solution was adjusted to a pH of 4.65, and a total molarity of 0.06 by adding sodium acetate (0.02M)-sodium chloride (0.08M) buffer. Cold 95% ethanol was then slowly added to this solution at 0°, with constant stirring, to obtain a final ethanol concentration of 50%. After standing for several hours in the cold, the precipitate was removed and discarded. Orosomucoid was precipitated from the supernatant by raising the concentration of ethanol in the solution to 80%. The final precipitate of orosomucoid was collected by centrifugation, dissolved in water and dialyzed against distilled water. The orosomucoid was lyophilized and stored at -20°.

Preparation of Exogenous Glycoprotein Acceptors

The acceptors for galactose were prepared both from fetuin and orosomucoid by removing terminal sialic acid and galactose residues. Sialic acid was removed from the glycoproteins by acid hydrolysis at pH 1.0 (10 mg protein/ ml of 0.1 N H_2SO_4 solution) for 1 hour at 80°. The release of free sialic acid was monitored by the method of Warren (283), and total sialic acid was measured by the method of Svennerholm (284). Acid hydrolysis removed about 98% of the sialic acid residues present in the glycoproteins. After hydrolysis, the solutions were neutralized with 0.1 N NaOH, dialyzed exhaustively against distilled water at 4°, and lyophilized.

The dialyzed glycoproteins were dissolved in 0.01 M potassium phosphate, pH 7.0, (2 mg protein/ml buffer) and were incubated with β - galactosidase (50 units/mg glycoprotein) at 37° under toluene for 200 hours. The release of free galactose was measured by the galactose oxidase method (Galactostat: Worthington). The procedure removed approximately 70% of the theoretical amount of galactose in fetuin or orosomucoid. After incubation, β-galactosidase was precipitated from the reaction mixture by the addition of perchloric acid to a final concentration of 0.34 N. The precipitate containing β-galactosidase was discarded and the supernatant was neutralized with 0.5 N KOH. The potassium perchlorate precipitate was removed and the solution was dialyzed against distilled water and lyophilized.

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