SOME ASPECTS OF GLYCOSYLTRANSFERASE ACTIVITIES IN LUNG TISSUE

ADRIAN BRETT SPRING

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SOME ASPECTS OF GLYCOSYLTRANSFERASE ACTIVITIES IN LUNG TISSUE

by

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A Thesis submitted in partial
fulfillment of the requirements for
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ABSTRACT

In our laboratory high sialyl- and galactosyltransferase activities were observed in lung homogenate.A differing behaviour using the detergent Triton X-100 was also observed for these enzymes. Following this the various properties and kinetic parameters of the two glycosyltransferases were studied in detail in lung microsomes. The optimum pH (6.8), optimum exogenous acceptor protein requirements (DS- and DSG- fetuin, 250 µg) and a linearity with time and protein concentration were established for sialyl- and galactosyltransferase. In addition, galactosyltransferase required Mn^{2+} (12.5mM) and ATP(2mM) for optimum activity. Sialyltransferase catalyzed the transfer of 2.3 nanomoles sialic acid to fetuin acceptor per mg protein per hour, and its apparent Km for CMP-sialic acid was 0.33mM. Galactosyltransferase catalyzed the transfer of 14.0 nanomoles of galactose to fetuin acceptor per mg protein per hour, and its apparent Km for UDP-galactose was 0.053mM.

Inclusion of the detergent Triton X-100 caused an 8-fold increase in the activity of galactosyltransferase and the V_{max} rose 6—fold, from 0.33 to 2.0. Sialyltransferase, in contrast, showed no dose—dependent response to Triton X-100 and was virtually unresponsive to Triton even in small

dose. Little change in the V_{max} of this enzyme in the presence of Triton was seen. Lysolecithin caused a specific activation of galactosyltransferase when compared to other lipid species added in a quantity considered to be physiological. Sialyltransferase, however, showed a general inhibitory response to all lipid classes tested. An additive effect was found on the glycosyltransferases when Triton and lipid were added on an equimolar basis. When the Triton dose was much greater than that of the lipid, the effects of the latter were masked by Triton. The one exception to this trend was found with LysoPA (oleoyl) whose effect on either enzyme's activity was not completely masked by the Triton.

An enrichment of both sialyl- and galactosyltransferase was obtained from purified lung surfactant and lung lamellar bodies. During the isolation of lung surfactant, sialyl-transferase was found in soluble form and galactosyltransferase was localized in membrane-rich fractions. Major proteins of molecular weights 64,000, 35,000, and 23-28,000 were identified in lung surfactant and lamellar body fractions, as revealed by SDS-gel electrophoresis.

A difference in the membrane localization and soluble nature of sialyl- and galactosyltransferase in lung tissue is discussed, with specific reference to the role of lysolecithin in this system. The role of glycosyltransferases found in lung surfactant and lamellar bodies may also involve membrane lipid changes in affecting the function of these enzymes. Possible relationships between glycosyltransferases and membrane phospholipids are discussed.

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ATP	adenosine triphosphate
Å	angstroms
BSA	Bovine serum albumin
Ca^{++}	calcium
cAMP	cyclic adenosine monophosphate
CDP-choline	cytidine diphosphate choline
cGMP	cyclic guanosine monophosphate
CMP-sugar	cytidine monophosphate sugar
CoA	coenzyme A
Co_2	carbon dioxide
CPT	choline phosphotransferase
DPPC	dipalmitoyl phosphatidyl choline
DPMC	1,palmitoyl,2-myristoyl phosphatidyl cholin
DS-fetuin	sialic acid depleted fetuin
DSG-fetuin	sialic acid and galactose depleted fetuin
DS-α_1-acid glycoprotein	sialic acid depleted α_1-acid glycoprotein
DSG-α_1-acid glycoprotein	Sialic acid and galactose depleted α_1-acid glycoprotein.
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethyleneglycol-bis (β-aminoethyl ether) N,N'-tetraacetic acid
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
G-6-Pase	glucose-6-phosphatase
MES	(2-[N-morpholino]) ethanesulfonic acid
O_2	oxygen
Mg^{++}	magnesium cation
$Na\ BH_4$	sodium borohydride
PA	phosphatidic acid
Lyso PA	Lysophosphatidic acid

PAS	periodic acid Schiff
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine.
PTA	phosphotungstic acid
RDS	respiratory distress syndrome
RER	rough endoplasmic reticulum
SDS	sodium dodecyl sulphate
SER	smooth endoplasmic reticulum
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylene diamide
TKM buffer	50mM Tris-HCl, pH 7.5, containing 0.025 KCl and 5mM $MgCl_2$
TM	tubular myelin
Tris	tris (hydroxymethyl) aminomethane
Triton	triton X-100, polyoxyethylated octylphenol
UDP-sugar	uridine-diphosphate sugar
dH_2O	distilled water
g	refers to g_{av} centrifugational force

INTRODUCTION

1. GLYCOPROTEINS

A. GENERAL

The last few years have seen a tremendous increase in interest in, and understanding of, the biochemistry of membranes. One membrane associated activity is the enzymatic glycosylation of complex polysaccharides, glycolipids and glycoproteins. Glycoproteins are naturally occuring compounds in which carbohydrates and proteins are covalently linked. Their chemical composition reflects the whole spectrum of the natural monosaccharides and amino-acids and molecular weights range from 16,000 to several millions. Glycoproteins occur either as soluble secreted materials or as intracellular membrane components. They are also important components at the periphery of living cells. They are currently considered to be cell-surface receptors to several biological effectors, including some hormones, viruses and mitogens. Roles in immunoprotection, cell-cell interactions in growth and differentiation of both developing embryonic tissues and adult tissues have been proposed. Several glycoproteins have enzymatic activity, some have blood-group specificity, and others have a structural role. The significance of the carbohydrate moiety in these compounds is not yet clear. In some it provides a part, or possibly all, of the functional site. In others its presence is probably more directly related to the localization of the protein portion and possibly to its rate of turnover.

A major group of glycoproteins formed in this way is that consisting of oligosaccharides linked to protein through a β-N-glycosidic linkage between N-acetyl-glucosamine and the amide nitrogen of an asparagine residue of the polypeptide chain. Other glycoproteins may be linked O-glycosidically to peptide bonded serine, threonine or hydroxylysine residues. There are basically two general types of oligosaccharide units involved: the simple and the complex type. The former group usually contains two N-acetylglucosamine residues proximate to the asparagine, with the second residue usually carrying a mannose linked to further mannose residues to give a branched structure. The complex types have a similar, though generally smaller core of mannose and N-acetylglucosamine residues and attached to this are small side chains made up of N-acetylglucosamine, galactose, and either sialic acid or fucose residues. The oligosaccharide portion of these molecules may play an important role in their localization in the cell, their secretion and in their turnover.

B. SYNTHESIS - ROLE OF GLYCOSYLTRANSFERASES

It is generally accepted that the protein moiety of these glycoproteins is synthesized in the ribosomes of RER. The ribosomes are on the cytoplasmic side of the membrane of the ER and in some way, as yet not completely understood, the proteins formed there that are to be secreted, permeate the

membrane to reach the lumen. During progress through the lumen of the SER and through the cisternae of the Golgi apparatus and secretory vesicles, progressive glycosylation occurs by a stepwise addition of monosaccharides to the polypeptide backbone as it moves through membrane channels on its way out of the cell. (Fig:I). The glycosyltransferases comprise several families of enzymes each of which catalyzes the following reaction:

sugar-nucleotide + oligosaccharide-acceptor ⟶
sugar-oligosaccharide-acceptor + nucleotide.

Thus, chain elongation of the oligosaccharide units in the complex carbohydrates is effected by the addition of monosaccharide units through the action of different glycosyltransferases in a specific sequence. The product of one enzyme reaction becomes the acceptor for, and the determinant of the next reaction. The sites at which sugars are added to growing carbohydrate chains are directly related to the positions these occupy in the overall carbohydrate sequence. Each heterosaccharide structure assembled within a cell of a particular genotype is specified by the mixture of glycosyltransferases present in that cell. The multiglycosyltransferase systems are arrayed along physically separate assembly lines, i.e, in different membrane compartments of the same cell. An extension of this idea is that the glycosyltransferases are actually required for secretion of glycoproteins and for their movement through the cell's

LEGEND FOR FIGURE I

The abbreviations are:

RER	=	rough-surfaced endoplasmic reticulum
SER	=	smooth-surfaced endoplasmic reticulum
G	=	Golgi apparatus
SV	=	secretory vesicle
PM	=	plasma membrane
EC	=	extracellular space
$T_1, T_2, T_3, \ldots T_n$	=	membrane-bound glycosyltransferases

The two kinds of discontinuous lines represent two types of glycoprotein undergoing synthesis: one type of glycoprotein is within the cisternae of the membrane system and is eventually secreted, whereas the other type remains membrane-bound throughout the biosynthetic process and is eventually incorporated into plasma membrane. The arrows radiating from the glycosyltransferases represent the process of glycosylation.

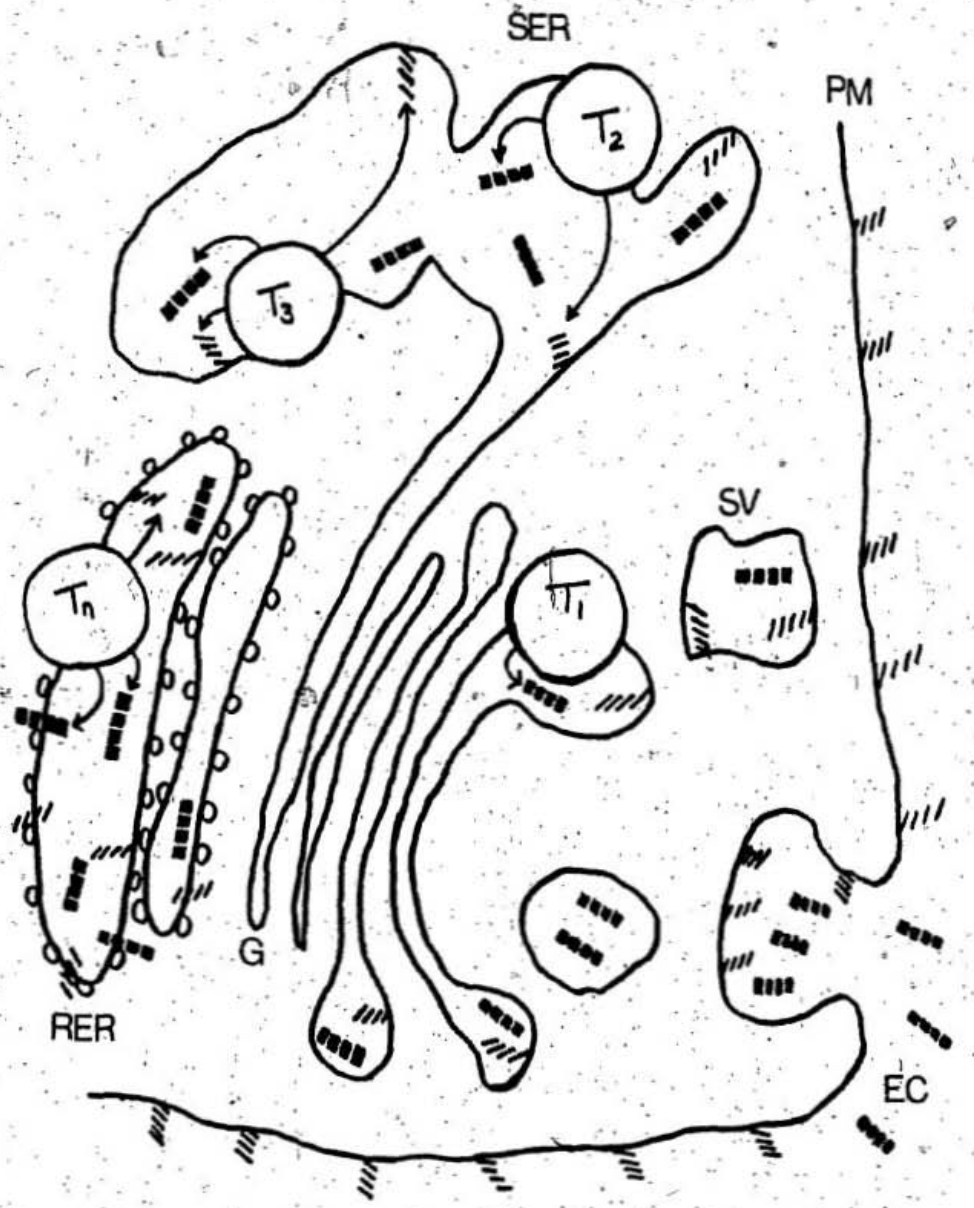


FIGURE I. Diagramatic Representation of Glycoprotein Synthesis in Liver Cell (taken and modified from Schachter (84).

membrane system. In other words, they are involved in the secretion of proteins into the circulation.

The membrane glycoproteins synthesized on membrane-bound ribosomes are arranged at the cisternal face of the RER, but still firmly fixed to the membrane. The transformation of membranes that contain newly formed components into SER then takes place. Secretory, transport vesicles derived from the Golgi form associations with the SER. The Golgi vesicles then fuse with the plasma membrane by reverse pinocytosis and contribute to the expansion of the total cell surface. In this way the biogenesis of cellular membranes is brought about by the transfer of membrane glycoproteins and glycosyltransferases from one cell compartment to another. The intracellular membranes are, therefore, chemically altered during passage through the cell due to the glycosylation of glycoproteins destined for incorporation into the plasma membrane (32). The overall compositions and sugar sequences of soluble, secretory glycoproteins and membrane glycoproteins are similar. They share the same mechanism of carbohydrate chain assembly and glycosyltransferase involvement. The biogenesis of surface membrane is, therefore, considered to be analogous to the secretory process. It is interesting that sialic acid and fucose, as terminal residues on the oligosaccharide chain, may have special significance in the recognition mechanism for membrane renewal and also in the process of exocytosis.

The study of the glycosylation of glycoproteins is, therefore, concerned with two important phenomena:

(i) the biosynthesis of glycoproteins destined for export from the cell,

(ii) the biogenesis of plasma and intracellular membranes.

Several experimental approaches have been taken to understand these processes:

(i) kinetic studies by pulsing intact cells with various radioactive precursors and following the incorporation of label into protein and lipid, by either autoradiography or biochemical analysis,

(ii) subcellular localization studies on the variety of glycosyltransferases involved in glycoprotein assembly,

(iii) static studies on the glycoprotein composition in subcellular organelles with respect to transformation of membranes within the cell.

These studies have resulted in a hypothesis of glycoprotein synthesis. The most fully studied of the glycosylation steps are those resulting in the addition of the side-chain sugars of the complex type of glycoprotein. The transferases responsible occur mostly in the membranes of the RER and SER and can be solubilized and, in certain cases, purified. They exhibit a high degree of specificity for sugar donor and glycoprotein acceptor. This, in turn, controls the sequential chain elongation and sequencing of these

oligosaccharides.

The preferred donor forms of N-acetylglucosamine and galactose are the uridine diphosphate sugars, whereas guanosine diphosphate fucose and cytidine monophosphate sialic acid donate the terminal fucose and sialic acid. With regard to acceptor specificity the N-acetylglucosaminyltransferase requires a glycoprotein carrying an oligosaccharide group with a terminal mannose residue to which the GlcNAc is attached by a β-glycosidic linkage. The appropriate galactosyltransferases catalyze the formation of a β-glycosidic linkage between galactose and a GlcNAc residue already linked to a mannose of an oligosaccharide. The sialyl and fucosyltransferases function most efficiently with a galactose acceptor already attached to a GlcNAc and mannose. While all glycosyltransferases within one family, such as the sialyltransferases, utilize the same sugar-nucleotide as the glycose donor (CMP-sialic acid), each of these enzymes is specific for the acceptor molecule or its analogues. Some enzymes, such as sialyltransferase, are strongly influenced in their rate by the nature of the penultimate sugar and by the position of the carbon atom in this sugar to which the galactose is attached. Some transferases that catalyze the transfer and binding of a sugar to peptide-bonded amino acids also exhibit a high specificity for stretches of the amino acid sequence that contain the acceptor amino acid.

Thus, these transferases operate sequentially in a specific manner during the movement of protein from the SER through the Golgi apparatus to the secretory vesicles and plasma membrane. The Golgi is responsible for attachment of the outer sugars of the complex glycoproteins. The transfer of proximal N-acetylglucosaminyl and mannosyl residues, the core sugars, is less understood. Autoradiographic studies in rat liver and biochemical studies on subcellular fractions show that these sugars are added mainly in the RER, and are incorporated, at least in part, while the polypeptide chain is still attached to membrane-bound ribosomes (81). Clarification of biochemical aspects is complicated by the difficulty in solubilizing and purifying the transferase activity from RER. The fact that these core-sugar transferases present in microsomal preparations will not function with exogenous putative acceptor proteins, but only with the small amounts of endogenous acceptor protein found here, also limits studies in this area. Although some direct transfer of N-acetylglucosamine and mannose to proximal positions on endogenous glycoprotein from appropriate-nucleotide donors has been observed, the transfer from lipid-soluble donors by lipid intermediates such as dolichol monophosphate has been more clearly demonstrated (31).

C. CONTROL OF GLYCOPROTEIN SYNTHESIS

The synthesis of oligosaccharide prosthetic groups is controlled by a non-template mechanism in which genes code for a large variety of glycosyltransferases. The post-ribosomal

steps for glycoprotein biosynthesis are not directly coded by a nucleic acid template. It is, therefore, conceivable that the existence and physical arrangement of batteries of separate glycosyltransferases of different specificities and variations in substrate availability in the membranes could serve as control loci for the rapid physiological regulation of glycoprotein biosynthesis and secretion. The nucleotide diphosphate sugar donors are formed in, or transported to the cytoplasm on the outside of the membrane, while the growing glycoproteins are situated on the inside of the membrane. The location of the transferases within the membrane is not yet clear, although adequate evidence suggests they are tightly bound to the ER and Golgi membranes. If the enzyme is also on the inside of the membrane, the donor has to permeate the membrane. The rate of this process will possibly change with the dynamic nature of the membrane. Alternately, if the enzyme bridges the membrane, the enzymic reaction itself may result in the transfer of sugars across the membrane and the question of permeability will not be critical. Obviously, the cellular levels of nucleotide-sugars, their access to the membrane sites and their alternative metabolic pathways (i.e. hydrolysis of UDP-sugars by nucleotide pyrophosphatase) will all act to control the rate of glycosyltransferase reactions.

The possible role of glycosyltransferase enzymes in the synthesis of secretory glycoproteins, in development, inter-

cellular adhesion and in surface recognition phenomena as related to transformation and growth, is well documented. However, in all these intra- and intercellular functions, glycosyltransferases are probably amenable to physiological regulation. To understand the purposeful and effective function of glycosyltransferases in vivo in regulating the sequential addition of glycosyl residues on the nascent and growing polypeptide molecule, it is essential to study the delicate micro-environment of these membrane-bound enzymes.

II. CONTROL OF MEMBRANE ENZYME ACTIVITY

The environment of an enzyme in free solution consists of water, substrates and other molecules in the solution. A membrane-bound enzyme also exists in a characteristic micro-environment, due to the effects of the membrane on which it is located, and therefore, its properties may be influenced by:

(i) general chemical and physical nature of the membrane, i.e. composition, lipophilic nature, charge, dielectric constant,

(ii) by specific interactions with individual molecules in its immediate neighbourhood, such as proteins, lipids, or carbohydrate,

(iii) by its intrinsic action on neighbouring molecules.

A. EFFECTS OF DETERGENTS

Membrane proteins can be termed either extrinsic or intrinsic, depending on how they are isolated from membranes. The extrinsic proteins are assumed to be bound to the membrane by mainly polar interactions with lipids or other proteins (34). The intrinsic proteins are tightly bound to the membrane, only being selectively solubilized by organic solvents or detergents, and they are presumably held by hydrophobic interactions. Extrinsic proteins can be considered to behave in a similar fashion to water soluble proteins in the presence of detergent, and may be released by detergents lipid-free. The interactions of detergent with intrinsic proteins, however, is more complex.

Helenius and Simons (30) have comprehensively reviewed the effects of detergents on the components of membranes, and the mechanisms by which detergents solubilize the bilayer. At prelytic concentrations of detergent a slight disruption of the lamellar structure of the bilayer occurs, allowing easier passage of small molecules across the membrane. As the detergent level is raised to lytic concentrations, the permeability barrier to macromolecules is lost, but the basic structure of the membrane is retained. Higher levels of detergent cause lamellar-micellar phase transition and thus solubilization into mixed micelles of detergent, lipid and protein. At very high concentrations of detergent, in which the binding capacity of proteins and lipids to detergent is fully saturated, the protein and lipid become separated into micelles of either detergent and protein, or detergent

and lipid. Additional detergent will cause size reduction of the micelles as the ratio of detergent to phospholipid increases.

Membrane solubilization can be assessed by determination of the ratio of detergent to membrane. This parameter is approximate, since it takes into account the total amount of detergent, rather than just that bound to the membrane. However, if the amount of membrane is high (approximately 2mg/ml) then free detergent will constitute only a small proportion of the total present. If this criterion is observed, then for Triton X-100 the necessary ratio of detergent to phospholipid for solubilization is 1.9 ± 0.9 (30). Quantities of detergent needed to reach a particular stage depend on membrane composition and on the detergent itself. The Triton X-100 to phospholipid ratio is an adequate parameter for the assessment of the extent of detergent-membrane interaction.

The action of detergents on membrane-bound enzymic systems is manifold. It can result in activation or inactivation of several enzymic activities, fragmentation of subcellular particles and solubilization of membrane-bound enzymes. To solubilize membrane glycosyltransferases one must get them out of hydrophobic environments to one where hydrophobic interactions are not possible, except between themselves. Alternately, it may be possible to substitute one hydrophobic environment i.e., membrane interior, for another, such as a mixed detergent micelle.

The detergent-protein complexes formed depend on the nature of the detergent used. SDS binds to both peripheral and integral proteins, which therefore usually undergo drastic conformational changes and a loss of biological activity. Triton X-100, in contrast, interacts predominantly with those proteins which are bound to the membrane lipids by hydrophobic interactions and binds to the hydrophobic part of the amphiphilic protein. At high Triton X-100 to phospholipid ratios, the more hydrophilic enzymes are susceptible to inactivation. No major conformational changes or loss of activity usually occur with the more hydrophobic enzymes in close association with lipid. These enzymes may show a stimulatory effect by Triton X-100 (where the enzyme is normally protected by its strong binding to the lipid). Only a part of bound detergent molecules interacts directly with the protein and the rest bind co-operatively to form a micelle-like region on the surface of the protein. The orientation of the protein (i.e., hydrophobic and hydrophilic parts) in two different phases is preserved during solubilization. The protein-bound detergent mimics the lipid environment in the membrane so that the protein activity is preserved. The efficiency of the action of Triton X-100 on the activity of membrane-bound enzymes depends on both the detergent/protein ratio and on the concentration of protein in the reaction mixture.

The effect of Triton X-100 on membrane enzymes is sometimes biphasic: activation is observed at low detergent

concentrations and inhibition at higher. Optimum activation usually occurs when the enzyme is still membrane bound. (By causing an unfolding of the membrane protein chains, Triton thereby promotes the accessibility of the substrate to the active enzyme sites for interaction). Only in a few cases where high concentrations of mild detergents cause delipidation of the membrane protein, does a loss of activity result (i.e. removal of phospholipids necessary for normal enzyme activity). By the behaviour of the enzymes to various Triton X-100 doses, one can infer something about membrane placement, i.e. how deeply embedded the enzyme may be in the lipid matrix. However, it is possible that exogenous detergents such as Triton X-100, which are normally added to in vitro assays of membrane-bound glycosyltransferases, extensively alter the organization of the lipid-protein matrix of the membranes and thereby obscure the attempts to study the in vivo physiological regulation of these enzymes. It is known that at excessive Triton concentration, membranes, bilayers and artificial liposomes, become more sensitive and fragile (33). Naturally occurring lipids such as lysolecithin, when added to in vitro enzyme assays, however, do not produce such drastic effects on similar systems.

B. EFFECTS OF MEMBRANE PHOSPHOLIPIDS

Despite its obvious importance, relatively little is known about lipid-protein interaction in biological membranes. Membranes are dynamic, constantly changing in the nature

and distribution of its components. Membrane fluidity can be affected by different combinations of naturally occurring phospholipids in the membrane, and it is known that the topographical distribution of phospholipids differs in different membranes. The appropriate lipid fluidity in the membrane is clearly important for the correct functioning of membrane-bound enzymes, which must possess the correct conformation in the membrane to perform its function.

There is quite strong evidence for the presence of a shell of immobilized lipid around at least some of the membrane-bound enzymes, and presumably these lipids are necessary for the activity of the protein.[20] The lipid requirement for some enzymes is rather non-specific, since many types of lipid can reactivate the enzymes to varying degrees - merely the availability of a hydrophobic region of the lipids is necessary. In others, more specificity is needed - both polar and apolar parts of the lipid molecule may be involved in influencing the conformation of the enzyme. Phospholipids with similar polar groups, but different fatty acid compositions have physical properties which reflect the properties of their fatty acyl moities. For example, lipids with longer and more saturated fatty acids (less fluid) are probably less effective in influencing enzyme functioning. Some enzymes require certain lipid head-groups, and may even require a certain fatty

acid pattern. In vivo local change in the membrane lipid environment may prove to be a factor in metabolic control. Local removal and replacement of phospholipid head groups or fatty acids could exert an allosteric control on a membrane enzyme.

The exact effects of the phospholipid environment on membrane-bound enzyme catalytic activity (proper conformation of active sites) may vary with different enzymes located at different topographical areas in the membranes, and therefore, each should be considered separately. An understanding of this relationship for each enzyme is essential for elucidating mechanisms of action and physiological regulation, the significance of membrane location, and the ways in which activity can be altered by various agents.

The evidence in favour of a specific role of phospholipids in the regulation of membrane-bound enzymes, is based on two different experimental approaches. In one type, the membranes are first treated with phospholipase A, or C, causing inactivation of the membrane-bound enzyme, and then the demonstration of an obligatory requirement of phospholipids for the reconstitution of the enzyme back to an active form is given (24). Membrane-bound ATPase, glucose-6-phosphatase, cytochrome oxidase and UDP-gluco-ionyltransferase belong to this category. These enzymes require rather harsh treatments with organic solvents in order to dissociate

them from lipids. Several lines of evidence indicate that phospholipids can be important, not only for maintaining the proper conformation of the active sites of certain membrane enzymes, but also for their stability. For example, it has been postulated that microsomal enzyme acyl-CoA-L-glycerol-3-P-acyltransferase requires phospholipids for normal catalytic activity, based on evidence that treatment of microsomes with phospholipase A and C leads to a decline in the activity of this enzyme, which, it is reported, is restored by phospholipids (2).

However, it is not possible to state that there is an absolute requirement for phospholipids. Since phospholipase A and/or C which inactivate these enzymes are specific in regard to their effects on the chemical bonds of the phospholipid molecule, interpretation regarding the specific interaction between the phospholipid molecule and enzyme protein has remained mostly speculative. For example, detergents, as well as phospholipids, restore to normal the activity of phospholipase C-treated ATPase (54). In most situations, reconstitution of the enzyme by phospholipids is non-specific, since the native source, base or fatty acyl moieties of phospholipids are usually of no significance to reactivate the enzyme. It may be that rather than inactivating through direct action, hydrolysis of phospholipids by phospholipase A leads to unstable forms of some microsomal enzymes. More extensive studies have been done on the regulation of mem-

brane-bound enzyme in this regard. Phospholipase A, inactivated G-6-Pase, but there was no correlation between the hydrolysis of phospholipids and the loss of enzyme activity, as the addition of EDTA completely stopped the hydrolysis of phospholipids by phospholipase A but did not oppose the decrease of G-6-Pase activity. The authors concluded that phospholipase A treatment produced an unstable form of the enzyme (105). Phospholipase A treatment is also known to labilize, but not inactivate directly, acyl-CoA-1-acylglycerophosphorylcholine acyltransferase in rat liver microsomes.

Experiments like these in which particular lipids are removed from membranes by the action of phospholipases, detergents or organic solvents, tend to be contradictory. Sometimes a particular lipid is reported to be necessary for an enzyme's activity, and other times it appears not necessary. The problem lies in deciding whether the inactivation of the enzyme is due to the removal of one specific lipid, or, due to the disruption of the membrane structure, as a result of the lipid removal.

C. LYSOLECITHIN EFFECTS ON GLYCOSYLTRANSFERASES

In the experiments of the second type, direct addition of phospholipids has been shown to activate a number of membrane-bound enzymes. Mookerjea et al (63-66) investigated the effect of phospholipids and other lipid factors on rat liver glycosyltransferase activities in the absence

of detergents. While enzyme activity was barely detectable in the absence of detergents, addition of exogenous lysolecithin caused a marked stimulation of ER or Golgi membrane-bound galactosyl-, N-acetylglucosaminyl- and sialyltransferases into endogenous or exogenous acceptor proteins. In contrast to the other membrane-bound enzymes described earlier, the lipid requirement of membrane glycosyltransferases was shown to be remarkably specific for lysolecithin, when compared to a number of other phospholipids and their lyso-derivatives. Lecithin, lyso PE, lyso PA and PG did not activate the enzyme, suggesting that both fatty acyl and phosphorylcholine groups of the lysolecithin molecule are required for the observed activation. The degree of stimulation was similar when myristoyl-, palmitoyl-, oleoyl- or stearoyl-lysolecithin were tested, and this activation was observed well within the physiological concentration of lysolecithin in the rat liver cell. The normal level of lysolecithin here is approximately 3mM (1.5 mg/ml), assuming its uniform distribution within the cell, although this concentration would be much higher in the membrane. When the dose effect of lysolecithin was studied in the presence of a saturating amount of Triton X-100 the stimulatory effect was completely masked. This would explain why the lysolecithin activation of these glycosyltransferases was not observed before, when Triton X-100 was included in the assay system.

Low concentrations of lysolecithin affect most membrane-

associated enzyme activities in one direction or the other. The regulation of glycosyltransferases by phospholipids is probably mediated by a different mechanism from that of a number of other membrane-bound enzymes (20,29,54,59,63,66, 68,74,87,104,106). The amount of lysolecithin in the cell membranes of most mammalian tissues is determined by a phosphoglyceride deacylation-reacylation cycle in which membrane-associated phospholipase A_2 enzymes act on lecithin in the membrane to produce lysolecithin which, in turn, can be converted back to lecithin by membrane-associated acyl CoA: lysophosphoglyceride acyltransferases (catalyze the transfer of fatty acids from CoA esters to lysolecithin). Therefore, the concentration of lysolecithin in different membrane locations may be further varied depending upon local differences in phospholipase A_2 and acyltransferase activities, (96). Brief pre-incubation of rat liver microsomes with phospholipase A_2 was found to stimulate glycosyltransferase activity, dependent on a pre-incubation lag time and an alkaline pH favourable for the hydrolysis of membrane phospholipid (63-66).

Several lines of evidence suggest that the stimulatory activity of lysolecithin on glycosyltransferases is probably derived from its detergent properties, which result from the presence in the same molecule of both a hydrophilic region (sn-glycero-3-phosphoryl-choline moiety) and a hydrophobic region (fatty acid ester moiety) with the appropriate

balance of hydrophilicity and hydrophobicity (87). The solubilizing effect by lysolecithin producing a micromiceller environment would facilitate the interaction between the enzyme and the water soluble nucleotide-sugar substrate (86). Both Triton X-100 and lysolecithin have been found to work synergistically. An approximate additive stimulation of transferase activity was observed with a mixture of equal weights of lysolecithin and Triton X-100 in the range of suboptimal detergent concentrations, again illustrating that lysolecithin effects are due to the surfactant properties of the entire molecule.

The high specificity of membrane glycosyltransferases in their lipid requirement can be further illustrated. The state of membrane fluidity (affected by different cation concentrations and different fatty acyl chain lengths of lipids) can be affected differentially by different combinations of naturally occurring phospholipids in the membrane, which, in turn, may regulate the membrane-bound glycosyltransferase activity. For example, PI and PS, two acidic phospholipids, are found to decrease galactosyltransferase activity in both lysolecithin and Triton X-100 treated microsomal micelles (67). However, raising the Triton X-100 concentration to a high level caused an enhancement of the Triton X-100 stimulation by PI and PS. Electron microscopic studies suggest that PI can effectively oppose the fluidization (solubilization) of the membrane components by

lysolecithin, and therefore retain the vesicular organization of the membrane concurrent with a restraining effect on the enzyme. PI and PS interact with divalent metal-ions and cause an increase in the packing density of the lipid fatty acid chains. At high Triton doses, where membrane bilayers become more sensitive and fragile, similarities of PI effects on lysolecithin and Triton-treated microsomes disappear.

We have seen how phospholipase A_2 and acylCoA: lysophosphoglyceride acyltransferases operate in a cyclic manner in membranes to control the level of lysolecithin. Lysolecithin generated can, in turn, greatly affect the general properties of the membrane such as fluidity and permeability. The levels of nucleotides such as cAMP and cGMP are known to regulate biological systems, such as hormone action, through opposing influences. These nucleotides are, in turn, regulated by the actions of adenylate cyclase and guanylate cyclase, respectively. Similar concentrations of lysolecithin have been found to stimulate guanylate cyclase and inhibit adenylate cyclase in mouse fibroblast microsomes, (97). This, therefore, suggests a model in which alterations of cyclic nucleotide levels in opposite directions can be achieved by the action of a single agent at the surface of the cell, i.e., hormone or mitogen → membrane [lysolecithin] → cyclic nucleotide response.

In conjunction with this it is interesting that cAMP and cGMP are found to prevent the precursor degradation in microsomal galactosyltransferase assays by the glycosyl-nucleotide pyrophosphatase. This effect leads to an overall enhancement of the transfer reaction, not by a direct stimulation of the enzyme, but through the maintenance of the substrate level. Pyrophosphatase inhibition is also observed with nucleotide tri-phosphates like ATP.

These enzyme modifications by lysolecithin, coupled with the observations that lysolecithin stimulates enzymes involved in consecutive steps of glycoprotein synthesis suggest that lysolecithin may play a wider role as a membrane transducer for the co-ordinate activation or deactivation of groups of functionally related membrane-associated enzymes involved in other cellular processes. Lysolecithin generated at one part of the cell could function as a transducer by diffusing rapidly through the lipid proteins of the cellular membranes to modify the activity of membrane-associated enzymes in other parts of the cell. In this context it is possible, therefore, that diffusion in the membrane plays an important role in the functioning of membrane receptors.

The occurrence of phospholipase A_2 in plasma membranes (99), the role of phospholipase A_2 and lysolecithin in the fusion of membranes (77,95) and the suggestions of Golgi and plasma membrane fusion in order to exteriorize secretory glycoproteins (14,77) may reveal further interlinked processes

between lysolecithin and glycosyltransferases. The glycosyltranferase enzymes have been recently shown to occur in human tissue fluids such as serum and amniotic fluid. The level of these transferases are found to increase, for example, in the serum of patients with liver disease (35-37, 62). It is possible that defective lysolecithin metabolism in the liver may be related to a hypersecretion and solubilization of these enzymes into the serum, and that membrane-bound enzymes are indeed solubilized and secreted by a physiological mechanism (involving phospholipase A_1 and lysolecithin).

III. THE LUNG

A. GENERAL

When considered in the context of the whole mammalian organism, the lung has one function, the transfer of O_2 from the environment to the blood and CO_2 from the blood to the environment. The body of knowledge regarding the functions of the lung has increased considerably during the last fifteen to twenty years. The report that more than forty kinds of cells have been identified in the lung clearly indicates the variety of metabolic activities and functions that are inherent to the whole lung. Since then, we have learned that the lung is a metabolically active organ, anabolizing and catabolizing pharmacologically active substances, as well as blood-borne compounds, that it synthesizes complex lipids, proteins and glycoproteins, and that it performs a defense function for the whole body. We also know that these functions directly and indirectly influence the respiratory function, which, in turn, affects the other lung functions.

B. RESPIRATORY DISTRESS SYNDROME

RDS of the newborn is a severe disorder of the lungs which is responsible for more deaths in the pediatric age group than any other disease. It is not so much a disease, as a consequence of developmental immaturity. Prior to birth the fetal lungs are filled with fluid. The net effect at birth is that some of the fluid is displaced and an air-

liquid interface is formed at the surface of the alveolar membrane. At delivery the infant must initiate breathing. The first breath normally requires very high intra-thoracic pressures to expand the lungs with air. The healthy term infant usually manages this without difficulty. On expiration after the first breath, the lungs normally retain up to 40% residual air of the total lung volume. Hence, subsequent breaths require far lower inspiratory pressures. The net effect of the liquid layer at the interface is an inward force tending to collapse the alveoli. This pressure (surface tension) is high during expiration, when lung air volume is low and therefore alveolar radius is decreased. The terminal alveoli are lined with surfactant material whose activity affects surface tension. For example, when the surface of the lung area is enlarged during inhalation, the increase in surface tension contributes significantly to the elasticity of the lung tissue, enhancing the organ's elastic recoil. Conversely, when the surface area of the lung decreases during exhalation, the surfactant brings about a sharp decrease in surface tension. This stabilizes the airways of the lung, prevents their collapse, and allows the organ to remain partially aerated. Surfactant functions are thus, two-fold:

(i) to decrease surface tension so that it requires less pressure to hold the alveoli open,

(ii) to maintain alveolar stability by varying surface tension with alveolar size.

In the premature infant who is developing RDS, the immature lungs are forced to take over the function of gas exchange before lung cells are able to maintain this function. Normal quantities and/or qualities of surfactant are not present in the alveoli of these children and the alveoli are, therefore, incapable of holding residual air, and they collapse with each breath. The same high inflating pressure is then required to initiate the next breath and, again the alveoli collapse on expiration. In effect, the infant is forced to take his first breath again and again and soon becomes exhausted. It is not exactly known whether the deficiency of surfactant in some way is due to a lack of maturation of enzymes responsible for surfactant lipid components, an inability to synthesize the apoprotein of surfactant, the synthesis of "defective" surfactant, an inability to secrete surfactant, or a combination of several of these factors.

C. SURFACTANT

Covering the single layer of epithelial cells over the basement membrane (the external cellular surface of the alveoli of the lung) is an acellular lining layer of variable thickness (100-1,000 A°) that extends over the entire alveolar surface. This is the only surface that normally comes into direct contact with alveolar air. Figure 2 shows that it is made of three components:

(i) the interface between the lining layer and alveolar air,

LEGEND FOR FIGURE 2.

ER = endoplasmic reticulum

G = Golgi

LB = lamellar bodies

M = monolayer

TM = tubular myelin

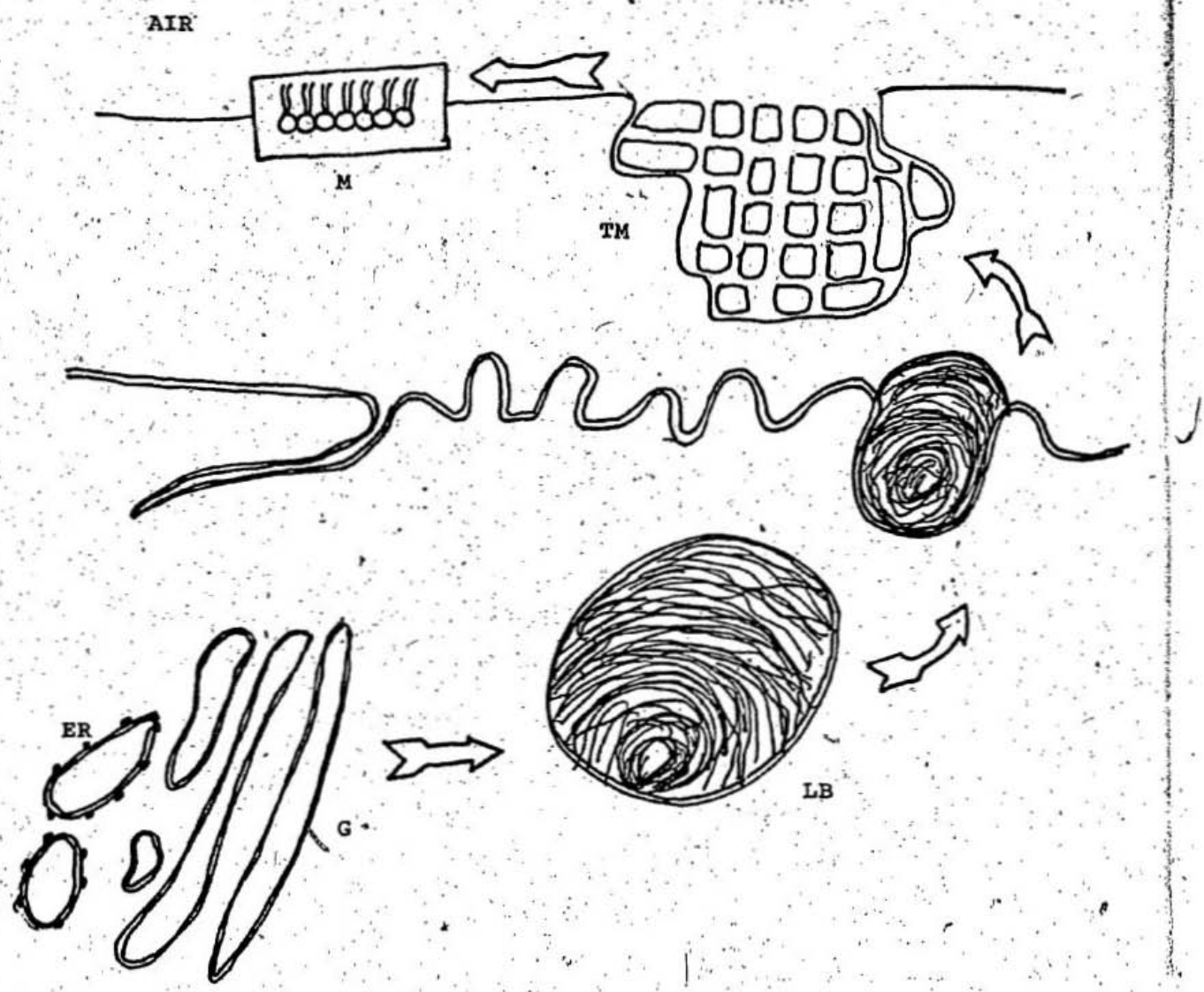


FIGURE 2. Schematic Representation of the Components of Surfactant Lining Layer and the Pathways of Secretion (29b).

the "surface film".

(ii) the bulk phase of the lining layer (hypophase) an aqueous subphase.

(iii) the interface between the lining layer and the cell surface.

The normal physical state and exact chemical composition of the alveolar lining layer has not yet been exactly defined, either quantitatively or qualitatively. Extensive research, however, indicates that certain phospholipids, proteins and carbohydrates, in addition to inorganic moieties, constitute the chemical matrix of this surfactant system. (Fig.3 A). Components of the surfactant system are produced in great part, and possibly entirely, by the type 2 epithelial cells (comprising about 6% of total cell population in the lung). These cells contain in abundance, not only the requisite organelles (mitochondria, substantial RER, Golgi and secretory vesicles) but also the enzymes necessary for the synthesis of lipids, proteins, and carbohydrates.

D. SYNTHESIS AND SECRETION OF SURFACTANT

Although we do not know the exact molecular basis for the synthesis, secretion and extracellular fate of surfactant, we do know the general sequence of events. Phospholipids and proteins are synthesized in the endoplasmic reticulum, perhaps modified in the Golgi cisternae, and then are transported to concentric, osmiophilic, membranous structures, the

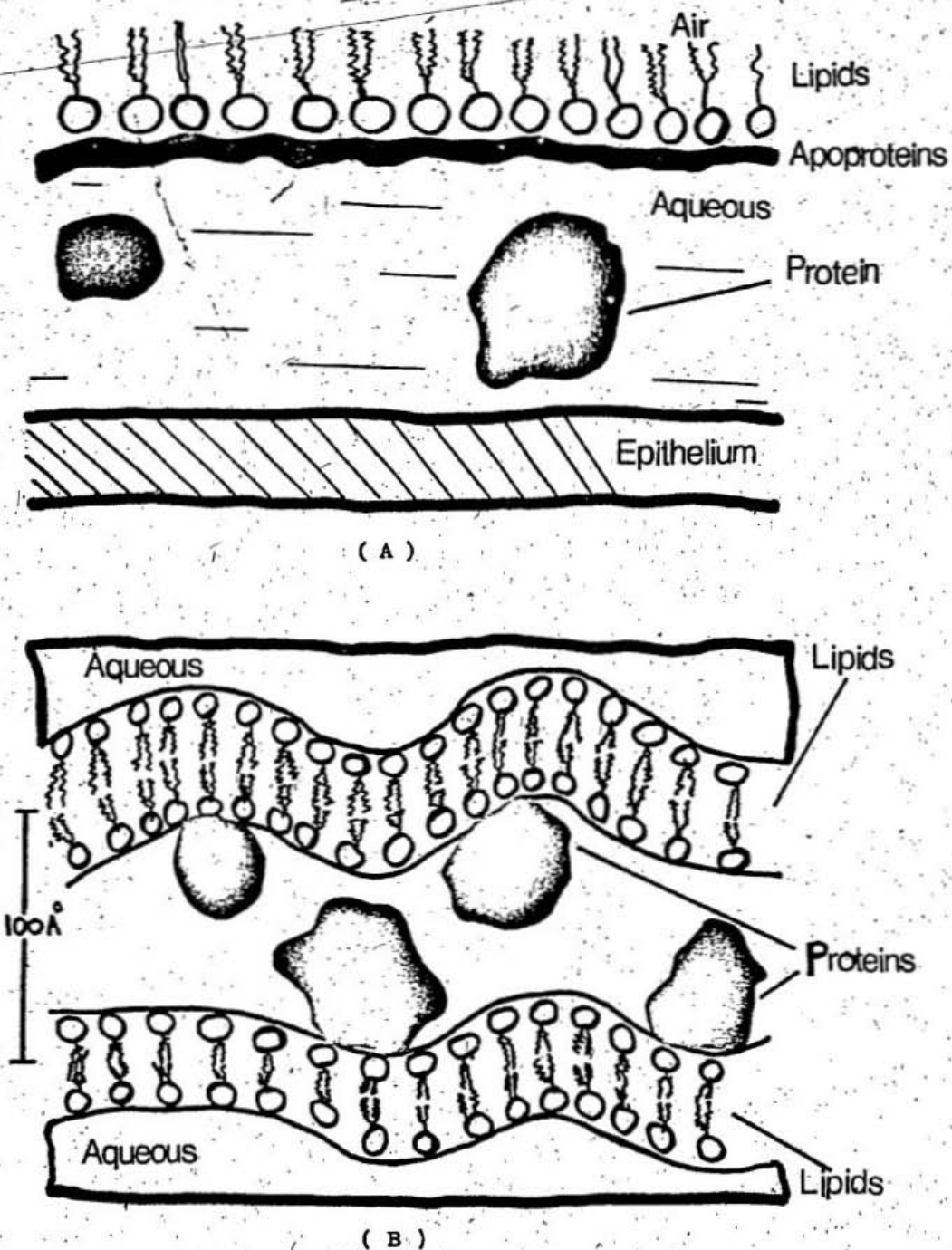


FIGURE 3. (A) Model of Surfactant Structure at Alveolar Surface. (98)

(B) Model of Lamellar Structure in Lamellar Body. (98)

lamellar bodies. These secretory granules, 1-2μ in diameter, develop from multivesicular bodies (pinched off vesicles from the Golgi). There is general agreement that lamellar bodies are the intracellular storage sites of surface-active material. They are secreted by exocytosis. Once outside the cell, lamellar bodies unfold to form tubular myelin (TM), a unique physical form of surfactant, appearing as layers of membranes arranged in a spherical granule (lattice structure). Gil and Reiss (27) isolated TM from rat lung and they have shown that it contains lipids similar to those in surfactant. The TM may be assembled by an active rearrangement of phospholipid and protein from a pool of building materials from the lamellar bodies. Finally, tubular myelin is adsorbed to the air-liquid interface to form the surface film (Fig. 2). In studies of developing lungs, the presence of alveolar surfactant has been correlated with the appearance and number of lamellar bodies found in the type 2 cells (27, 43, 59, 87).

E. COMPOSITION OF SURFACTANT

Of the three components of the alveolar lining layer, least is known about the chemical composition of the hypophase-cell boundary. Presumably, the surface of the epithelial cell plasma membrane is analogous to that of other cell membranes, probably a lipid-protein complex. It appears that the type 2 cell hypophase cell boundary contains a

high concentration of polysaccharides analogous to the characteristic surface coating, the "glycocalyx" of many cells (mucinous mucopolysaccharide and glycoproteins). Histochemical staining patterns and the easy digestibility with sialidase suggests a sialomucin in this layer (52). This material is found in the same lavage fraction as surfactant phospholipid, but its elaboration within, and secretion by the type 2 cells is different from surfactant phospholipid. It appears before the onset of surfactant or the lamellar bodies. It is thick just before birth, decreases with the onset of breathing in the newborn, and increases again in mature adults (53). The composition of the hypophase, however, is still a matter of much contemporary debate and speculation. The glycocalyx— like carbohydrate found here, may represent an active process of extrusion of cellular material. For example, an active turnover or shedding of glycoproteins is known to take place at cell surfaces. The type 2 cell has a thicker than average glycocalyx, still attached firmly to the cell membrane by hydrophilic forces, and leaving a large hydrophilic framework to extend relatively large distances into the extracellular space. With regard to the lung surfactant system, this hypophase region-glycocalyx may represent a well-adapted system in providing an area of restricted diffusion of metabolites near the cell surface, or perhaps as a protective filter system for surfactant secretion.

It is now known that the surfactant system is a complex association of lipid (primarily dipalmitoyl lecithin), protein and carbohydrate. Careful ultrastructural analysis of the conformation of the hypophase also indicates that it contains lipid, carbohydrate and protein, giving the appearance of a homogenous-somewhat granular-acellular covering over the epithelial cells (Fig.3A). The hypophase also contains sufficient concentrations of inorganic electrolytes (i.e. Na^+, K^+, Ca^{++}, Cl^-) to impart optimal surface activity to the organic surfactants of the lining layer. The purification of surface active material from endobronchial lavage fluid has been effected by most workers using differential and density gradient centrifugation in either salt or sucrose solutions (25-27, 3,48,82,105). This surfactant is the classic example of the maintenance of lung structure by macromolecules secreted by lung cells, and the importance of this to the survival of the organism provides for local and fine regulation of biosynthetic and secretory processes.

F. EVIDENCE FOR SYNTHESIS AND SECRETION

In recent years it has become evident that lung cells are capable of synthesis, intracellular transport, and active secretion of molecules which are likely to be components of the lung's surface active extracellular lining layer. A large body of work has shown that the lung can utilize various

substrates such as glucose, acetate, glycerol, and palmitate, to form phospholipids (12, 23, 72, 85). Glucose also augments *in vitro* protein synthesis in the lung. Glucose also is found to be the source of the carbon skeleton in lung epithelial glycoproteins (102). *In vivo* studies have indicated that after the administration of radioactive sugars and amino sugars, radioactive glycoproteins appear in the lung in moderate amounts compared to other organs (7, 79). The *in vitro* utilization of radioactive glucose for glycoprotein synthesis by the lung has been demonstrated, the radioactivity appearing as amino sugars, rather than as amino acids in protein (102). Lung slices can incorporate radioactive leucine into protein and, more specifically, into nonserum protein found in a surface-active lung fraction (19, 56).

Using lung lavaged alveolar type 2 cells radioactively labelled sugars were incorporated into protein at the microsome level with subsequent transfer of this to particles sedimenting at 15,000g (known to contain lamellar bodies). Simultaneous incorporation (synthesis) at two or more subcellular sites, but at different rates, was ruled out because the protein specific activity in the cell homogenate was constant throughout the incubation time, therefore illustrating real protein transport (55, 56). An *in vitro* study established the lung as the site of synthesis of the transported proteins, since immediately following a pulsed incu-

bation of rat lung slices with tritiated leucine, most of the grains (70%) in the granular pneumocyte appeared over the RER, with only about 11% over the lamellar bodies. After forty minutes, 50% of the grains were over the RER and 30% over the lamellar bodies. No change in the distribution of radioautographic grains with time was seen over the nuclei or mitochondria. Chevalier and Collet (11) in another radioautographic study, also using tritiated leucine, demonstrated a flow of radioactivity through the Golgi apparatus and the multivesicular bodies to the lamellar bodies. They also provided insight into the intracellular transport of lipid and glycoprotein precursors in the type 2 alveolar cells. Using radioactive choline they demonstrated that the grains were initially localized over the RER, then transferred through the Golgi apparatus into the lamellar bodies. Using radioactive galactose, they demonstrated an initial appearance of the silver grains over the Golgi (site of incorporation of galactose into exportable glycoproteins) and then over the lamellar bodies. Moreover, images of the discharge of labelled lamellar bodies into the alveolar cavity from these cells was observed. These three radioactive precursors should have a relatively high specificity for the phospholipid, protein and carbohydrate components of surfactant.

In vivo studies show that newly synthesized protein

is released by lung slices, and this release is inhibited by low temperature and cyanide, and stimulated by epinephrine and pilocarpine, indicating it is an active, energy requiring secretory process (56). In addition, the time course of the initial distribution of the in vivo administration of radioactive palmitate and proteins, between lung tissue and lung lavage returns, are very similar, and are consistent with a precursor-product relationship between DPPC and protein in both these fractions.

G. SURFACTANT LIPID

The phosphatidylcholines of lung are unique in that approximately 35% are disaturated. The usual type of PC found in animals and plants contains a saturated fatty acid in the Sn-1, or α position, and an unsaturated fatty acid in the Sn-2, or β position. Palmitate occurs in both positions in the commonest form of disaturated PC isolated from lung, DPPC, the main lipid found in purified surface active material. About 50% of the lipid found in surfactant is DPPC. Other lipids found here include cholesterol, triglycerides, phosphatidyl ethanolamine and sphingomyelin. Phosphatidyl glycerol is also highly surface active and is synthesized in the microsomes and secreted in the lamellar bodies. PG is the second most abundant phospholipid in surfactant.

The unsaturation of fatty acid chains of phospholipids increases the average area per molecule and, therefore, increases the intermolecular spacing between lipid molecules

(in monolayers). This influences the enzymic hydrolysis and interaction of divalent cations. Surfactant's activity appears to relate chiefly to its chemical configuration. Since the molecule contains a hydrophilic choline group and two hydrophobic saturated fatty acid side chains, it becomes oriented on the alveolar surface so that the saturated fatty acids form a highly stable, compressible, tension lowering film. In general, a separation between the hydrophilic and hydrophobic groups in a molecule causes it to orient at an air-water interface. The hydrophilic groups tend to dissolve in the acqueous phase, while hydrophobic groups stay out of the acqueous phase. Although both DPPC and DPMC species of lecithin are nearly equal in their ability to lower surface tension, β-palmitic lecithin is a much more effective surface-active agent than the myristic product because it is more stable. It can maintain its surface activity for about 5-8 times longer than β myristic lecithin (13, 47, 48, 69). Techniques to characterize surface properties and surface tension, using monomolecular films floated on H_2O in a surface balance are used to relate electromotive forces across the surface to changes in the composition and concentration of molecules in the film. The marked lowering of surface tension by surfactant is due to the packing of its lipid molecules into a close-ordered array at the interface, and thus is a mass-related property. When the interfacial tension is decreased there is a greater concentration

of molecules at the interface than the concentration in the bulk solution. Only DPPC has the necessary surface properties and exists in sufficient amount to lower the surface tension of the alveolar interface to less than 10 dynes/cm.

H. LIPID SYNTHESIS - FORMATION OF DPPC IN LUNG

Studies of fetal lung phospholipid synthesis are difficult to interpret because there are species differences in the length of gestation, enzyme maturation time-tables, and even in the composition of surface-active lecithin. Because techniques for the isolation and growth of alveolar type 2 cells in culture are now in the early stages of development (40,41,98), studies of the biosynthesis of surfactant phospholipid have, of necessity, been carried out in the past on tissue homogenates, subcellular fractions, or by *in vivo* uptake of labelled precursors. These activities cannot necessarily be equated with surfactant production *in vivo*.

Glycogen is probably a major source of carbohydrate substrate for PC synthesis in the alveolar type 2 cell. Alveolar cell glucose may be derived from glycogen breakdown, or from circulating blood glucose. Glucose may be catabolized to acetyl-CoA, a starting point for *de novo* fatty acid synthesis. Fatty acid synthetase in fetal lung produces palmitic acid. Lung fatty acids may also be derived

from circulating fatty acids. Compared to other tissues, lung tissue is very active in the general synthesis of lipids. In contrast to the liver which incorporates most of the fatty acids into triglycerides, the lung favours their incorporation into phospholipids. Glucose may also be converted into glycerol-3-phosphate. Circulating glycerol is rapidly incorporated into pulmonary PC. In the absence of glucose in the incubation medium, lung slices oxidize acetate to CO_2, whereas when glucose is present, acetate tends to be used for phospholipid synthesis. Two fatty acids combine with glycerol-3-phosphate resulting in the formation of a diglyceride after the phosphate moiety is removed by PA phosphatase. Choline is activated to choline phosphate by choline kinase and then converted to CDP-choline by choline phosphate cytidyltransferase. The final step in the de novo synthesis of PC is the interaction of diglyceride with CDP-choline by choline phosphotransferase. These main pathways of lipid synthesis, including fatty acid synthesis, acylation of glycerol and the CDP-choline pathways are localized in the microsomes.

There are several pathways for the synthesis of DPPC in the lung that have been demonstrated in vitro, but the pathway responsible for the synthesis of DPPC in surface active material remains speculative. The major questions are: Where are the two palmitates added, especially the palmitate in the Sn-2 position? Do they occur in the

initial PA or diglyceride, or is 1 added by altering a pre-existing unsaturated PC?

The four main pathways for PC formation in the lung all start with diglyceride or intact phospholipids. De novo synthesis of lecithin proceeds via the CDP-choline and N-methyltransferase pathways. The DPPC synthesis by a selective N-methylation of saturated species of PE has been found to be small, and no precursor-product relationship exists between the methylated intermediates and surface-active lecithin. The activity of this pathway also declines just before birth, while the concentration of surface-active lecithin continues to rise. In the fetal rabbit the composition of the alpha and beta carbon acyl esters varies with the time of gestation. Early in gestation, the concentration of palmitate is low, especially of the beta carbon. Between the 27th and 28th (31 day term) days the concentration of palmitate suddenly increases at both the alpha and beta position. In the CDP — choline pathway that we have already discussed, choline phosphotransferase is the only enzyme that can affect the species of lecithin formed. This activity is found to be quite high early in gestation, but then declines until term. In the past, the bulk of the evidence in the adult animal suggested that the major route of lung DPPC synthesis was by the CDP-choline pathway. Moriya and Kanoh (70) measured

the radioactivity in different species of phosphatidic acid, diglyceride, and phosphatidylcholine in lung at various times after the intravenous injection of [^{3}H] palmitate and [^{3}H] glycerol. There was a rapid rise in radioactivity in the disaturated species of PA and diglyceride, followed by a rise in the specific activity of DPPC. These findings agreed with the precursor-product relationship in the CDP-choline pathway. However, the distribution of [^{3}H] palmitate in diglyceride and PC was different, and the increased radioactivity in position 2 of DPPC could not be accounted for by the CDP-choline pathway. This discrepancy could be accounted for by other pathways of synthesis of DPPC or by different specific activities of substrates in different cell types within the lung. It is likely that, in whole lung, there are contributions by all pathways and that, in studies with isolated cell types, one particular pathway will dominate. In other tissues there is no data to show a selectivity of the utilization of diglyceride by CPT (91,103).

The third pathway for the formation of DPPC is by the acylation of lysolecithin. This pathway converts one species of PC into another by removing one fatty acid and re-esterifying it with another. In slices from rabbit lung there is evidence that acylation of lysolecithin forms DPPC and that unsaturated PC is converted to DPPC. Lung contains microsomal enzymes that can acylate 1-acyl-2 lyso PC and 2-acyl-1-lyso PC (25). The important feature of lung

microsomal enzymes, as compared to those of the liver, is the relative lack of specificity of acylation of the one and two position with saturated and unsaturated fatty acids (25). In liver, the acylation reactions form only unsaturated PC, whereas in lung, the acylation reactions can readily form DPPC (i.e., in lung, the fatty acid specificity of glycerol-3-phosphate acyltransferase is responsible for introducing saturated fatty acids at both the one and two positions). We know that, in the lung, the highest concentrations of DPPC are found in purified surface active material and in lamellar bodies (27,42). DPPC and PG are over 80% the total phospholipid in lung wash and lamellar bodies, but only about 50% of that in mitochondria or microsomes (21). Lamellar bodies are found to have acyltransferase activity and they are twice as active as mitochondria or microsomes in synthesizing DPPC from unsaturated PC preformed in the microsomes (5,60).

The fourth pathway found in the lung also converts unsaturated PC into disaturated PC. This pathway occurs in adult rat lung and in fetal and adult rabbit lung (4,101). This involves the trans-esterification of palmitate from 1-palmitoyl-lysophosphatidycholine by the lysolecithin-acyltransferase involving two molecules of lysolecithin, as described by Abe et al (1). Because most of the naturally occurring lysolecithin would be 1-palmitoyl 2-lyso PC,

this pathway is likely to synthesize DPPC *in vivo* as it does *in vitro*. Studies in both the lung and liver suggest that transacylation of lecithin fatty acid esters occurs at a faster rate than the de novo synthesis of lecithin.

A number of studies in recent years have further alluded to the reacylation-deacylation pathway for synthesis of DPPC found in lung surfactant. Possmayer *et al* (78) studied the acyl specificity of CDP-choline: 1,2-*sn*-diacylglycerol choline phosphotransferase to determine the effect of the degree of unsaturation of the fatty acids in the diacylglycerol on this enzyme's activity. With the exception of 1,2-dimyristoyl-*sn*-glycerol, the presence of diacylglycerols with saturated fatty acids at the *sn*-2 position did not produce a definite stimulation of CPT activity in rat lung microsomes above that observed with endogenous diacylglycerol. Under optimal conditions, using CDP [^{14}C] choline, rat lung microsomes CPT activity is markedly stimulated by exogenously added 1,2-*sn*-diacylglycerols containing an unsaturated fatty acid at the sn-2 position, especially if palmitate is the fatty acid in the sn-1 position. It has also recently been shown by van Golde (97) and by Rooney *et al* (80) that the preferred substrates for choline phosphotransferase are diglycerides containing unsaturated fatty acids, and therefore, the end product of de

novo synthesis is most likely unsaturated PC.

Snyder and Malone (90) used a homogenous population of alveolar type 2 cells (induced as lung adenomas by urethan) and found that in lung microsomes, in the presence of ATP, CoA and Mg^{++} 90% of the 1-^{14}C palmitate that is incorporated into 1-acyl PC is located at the 2 position, indicating that surfactant DPPC can readily be formed by insertion of palmitate at the 2-position of PC molecules, presumably by a deacyl-acylation enzymatic sequence involving 2-lyso PC. The control of acyl specificity at the 2-position is determined by the relative concentrations of the co-participating substrates, 1-palmitoyl-sn-glycero-3 phosphocholine and palmitoyl-CoA. Microsome preparations can catalyze significant incorporation of palmitic acid into the sn-2 position, independent of PA formation as evidenced by the fact that sn-glycerol-3-phosphate and calcium ions which inhibit choline phosphotransferase did not influence the incorporation of PA into DPPC. However, one must be extremely cautious in interpreting results obtained on the incorporation of specific fatty acids into surfactant PC, since the specificity varies with the substrate concentrations used.

Moriya and Kanoh (70) have reported that disaturated diacylglycerols account for 11% of the diacylglycerol pool in rat lung. Only 1.1% of the PC's produced from the endogenous diacylglycerols in rat lung microsomes were disat-

urated. The [^{14}C] dipalmitoylglycerol incorporated into PC was small, even when this diacylglycerol was dispersed with egg diacylglycerols. A novel exchange reaction was discovered in rabbit lung by Kyei-Aboage et al (49) in which free palmitate can exchange with an unsaturated fatty acid such as oleate at the 2 position of PC. The lung contains enough 1-palmitoyl--oleoyllecithin for this to be significant. Ca^{++} is required and phospholipase activity is probably involved. At present it is still not possible to decide which of these metabolic routes serves as the major pathway for the production of DPPC in lung surfactant. However, the observation that pulmonary CPT only produced limited amounts of DPPC in vitro from the endogenous pool of microsomal diacylglycerols or from those generated by phospholipase C suggests that DPPC may not be produced in vivo to any great extent by the de novo pathway for lecithin synthesis (88).

I. ISOLATION AND FUNCTION OF GLYCOPROTEIN IN LUNG SURFACTANT.

The chemical composition of pulmonary surfactant has evolved in a way that promotes the maximum effectiveness in its physiological function. It contains a sufficient amount (enough to cover alveolar surface with a monolayer) of DPPC so that a duplex film can reduce the surface tension of the alveolar interface to the low values required for stabilization of alveolar structure. (<10 dynes/cm). However, the physical state of lipids is, in part, determined by other lipid and protein compounds in their immediate milieu. Pure

DPPC is in a gel-like state at 37°C and its rate of absorption to an air-water interface is too slow to be physiologically important in providing low surface tensions and alveolar stability. It contains, in addition, components, apoproteins, which enable it to adsorb to the surface in times short enough for normal physiological requirements. It is found that pulmonary surfactant containing apoproteins adsorbs from a saline subphase to its air-liquid interface more rapidly than do sonicated lipids extracted from the same material(98). Thus, the apoproteins may accelerate the extracellular transport of pulmonary surfactant to the alveolar interface and, in this manner, insure that adequate amounts are available to the surface upon demand. A single species (constituent) is apparently not able to manage all these functions, and the chemical composition of surfactant thus represents a compromise between the two functions. The price that is paid for this increased molecular mobility is that the surface film does not have as high a stability as pure DPPC. Thus, the collapse rate is relatively rapid and, of course, the replacement rate must also be relatively rapid. On the other hand, the collapse rate is sufficiently slow at 37°C that surface tension in normal alveoli can remain very low and stabilize them for long enough intervals to permit adequate gas exchange to occur between the alveolar gas and pulmonary capillary blood(98).

It is thought that a reservoir of surfactant exists in the alveolar subphase and that part of this reservoir can adsorb readily to the alveolar surface as needed (in <1 minute). Randomly formed DPPC could be sequestered physically by apoproteins and allow surfactant molecules to re-enter the film during lung expansion and thus prevent any continual depletion of surfactant at the interface, (surfactants are expelled from surface monolayer during film compression)(98). These physical processes at the air-water interface could also control metabolic processes inside the type 2 cells, for example, the secretion of the lamellar bodies into the alveolar lumen. These apoproteins, however, do not seem to do much to the mechanical or electrical properties of the surfactant once the film has been generated(98). After secretion, the composition and structure of surfactant may change since large molecules tend to uncoil when they reach an air-liquid interface and proteins may be denatured.

Because of their insolubility in aqueous media, most lipids need a transport protein. The role of albumin in the transport of fatty acids and the role of serum lipoproteins in the transport of cholesterol ester and triglyceride are well known examples. Similarly there are proteins within cells like phospholipid-exchange proteins for transporting phospholipids. It is not unlikely that control of intracellular lipid metabolism may reside, not with lipids

and their precursors, but in the specificity and turnover of these proposed transport proteins. There is no data on intracellular transport proteins in the lung. This is one reason, however, for the interest in the apoproteins associated with surface active material.

King et al (43) isolated proteins from surface active material in lung lavage and studied their physical and chemical properties. Apoproteins obtained from canine, human and sheep sources were quantitatively precipitated in 1:3 ($^{v}/_{v}$) ethanol and ether and dissolved in aqueous solutions of 0.1% SDS, 69,000, 34,000, and 11,000 dalton proteins were resolved by polyacrylamide gel electrophoresis in SDS and 2-mercaptoethanol. The two lower molecular weight proteins were comprised of nearly 60% hydrophobic amino acid residues, making them comparable in hydrophobicity to proteins found normally in membranes that are poorly soluble in aqueous solutions and are thought to be embedded in lipid matrices. The surfactant protein showed a strong affinity for phospholipid and contained twice as many acidic as basic amino acids. Purified surfactant migrates as a lipoprotein through eight centrifugations, two continuous density gradients, isoelectric focussing in pH gradients, in starch gel electrophoresis and during six additional centrifugal flotations. This suggests that there may be large portions of the molecule made up exclusively of hydrophobic residues and these might interact with the fatty

acid moieties of the phospholipids. As measured by quantitative immunoassay these proteins are concentrated 50 - fold in pufified surfactant as compared to whole lung homogenates.

Passero et al (75) isolated two proteins from the lavage fluid obtained from human patients with alveolar proteinosis (the alveoli and terminal bronchioles are filled with PAS positive amorphous material). These proteins migrate in SDS polyacrylamide gel electrophoresis with molecular weights of 36,000 and 62,000 daltons. The uncertain etiology of this disease, which may be due to either the overproduction of surfactant by alveolar cells or, a defective clearing of the alveoli, provides additional interest in determining the origin of these proteins. Bhattacharyya et al (8) found these same two identical glycoproteins in particulate material obtained by pulmonary lavage from normal rabbits. Both have hydroxyproline and a relatively large amount of glycine (Table 1). Gel filtration and gradient centrifugation techniques yielded fractions from these two glycoproteins with galactose, galactosamine, fucose, glucosamine, and sialic acid (Table 1). The same two glycoproteins with the same amino acid and carbohydrate composition were also isolated from lamellar bodies obtained utilizing differential and density gradient centrifugations. In addition, similar results were obtained in the lavage and lamellar body fractions of dog and chicken (10). The unique, collagen-like glycoproteins are, therefore,

TABLE 1

AMINO ACID AND CARBOHYDRATE COMPOSITION OF PURIFIED GLYPROTEINS FROM RABBIT LUNG (8)

AMINO ACID	Lavage glycopeptides		Lamellar body glycopeptides	
	62,000	36,000	62,000	36,000
Aspartic acid	96.0	81.0	95.8	80.9
Glutamic acid	115.9	90.5	116.0	90.5
Threonine	51.7	47.6	51.6	47.4
Serine	48.2	48.6	48.4	48.7
Proline	53.6	62.0	53.5	62.0
Glycine	110.0	148.8	110.0	148.0
Alanine	76.2	71.0	76.1	71.1
Half-cystine	12.4	17.4	12.3	17.6
Valine	63.5	65.6	63.5	65.8
Methionine	14.0	10.6	14.1	10.6
Isoleucine	29.6	44.5	29.5	44.6
Leucine	86.0	96.4	86.1	96.6
Tyrosine	46.7	33.8	46.7	33.8
Phenylalanine	50.2	41.8	50.1	41.9
Lysine	63.0	42.5	63.1	42.5
Histidine	24.8	23.6	25.0	23.5
Arginine	50.3	58.4	50.6	58.4
4. Hydroxyproline	8.2	12.0	8.3	11.9
Hydroxylysine	0	0	0	0
NH-terminus	Valine	Threonine	Valine	Threonine

Amino acid compositions are expressed as residues/1,000 amino acids. The results are average of triplicate analyses of 24-h hydrolysates: no corrections have been made for destruction or incomplete hydrolysis.

Carbohydrate	Lavage glycopeptides		Lamellar body glycopeptides	
	62,000	36,000	62,000	36,000
	% carbohydrate/peptide			
Sialic acid	1.20	1.50	1.22	1.50
Mannose	1.50	2.00	1.50	1.95
Fucose	0.60	0.78	0.60	0.76
Galactose	1.00	2.20	1.00	2.20
Glucosamine	0.62	0.60	0.62	0.60

The compositions are expressed as percentage of carbohydrates per peptide. The methods of analysis are given in the next Results are average of triplicate analyses.

major proteins of avian airways (9). Because avian airways contain no Clara, ciliated, or goblet cells, but do contain type 2 cells as the only secretory airway cells with lamellar organelles, it is likely that in birds this glycoprotein(s) may be a product of type 2 cells.

J. ROLE OF LAMELLAR BODIES

Lamellar bodies are the storage or secretory form of the surfactant system, and these organelles appear simultaneously with surfactant activity in the prenatal lung. This suggests that lamellar bodies may be the source of these glycopeptides. They may also function to attach lipid, carbohydrate, and protein moieties of surfactant before their release into the alveoli air spaces. We know that the inclusion bodies contain material capable of lowering surface tension to less than 18 dynes/cm, and that it contains similar amounts of DPPC as surfactant (Fig. 38)(Ref). These bodies have been found to have relatively high specific activities for enzymes usually found in lysosomes—aryl sulfatase, β-D-galactosidase, α - mannosidase, acid phosphatase, β-glucuronidase and β-N-acetyl glucosaminidase(Ref). The function of these lytic enzymes here is still not understood. They may provide a catabolic system for the overproduction of secretory products such as surfactant. These enzymes are secreted along with the lipids of surface-active material. They may also function to clear materials from the alveolar surface or to alter the

functions of other lung cells, or to influence the physico-chemical state of accompanying phospholipid and, therefore, affect the surface activity of the latter. A possible effect on the glycoproteins associated with surfactant may provide further function to these enzymes, especially the glycosidases, as lamellar bodies are found to have a higher protein/phospholipid ratio than that of alveolar surfactant (19,103).

K. EVIDENCE FOR GLYCOPROTEINS IN SURFACTANT

The proteins found in surfactant appear to be unique to this lung material, and they are not found anywhere else in living tissue. As a means of specifically identifying and quantifying surfactant in biochemical and physiological experiments, King and collaborators (44) developed a radioimmunoassay for surfactant, capable of detecting 0.02 to 0.04 μg of apoprotein, and control experiments indicate that it is specific for surfactant. The competition for antibody binding sites by whole surface active material can be quantitatively accounted for in the assay by the binding of 34,000 dalton apoproteins.

Further evidence for the specificity of apoproteins in surfactant comes from the fact that they are not found in plasma or serum, and they have been localized at the alveolar interface by immunofluorescence. These apoproteins have been purified from surfactant using procedures designed to separate away soluble proteins (46). They are found in tracheal fluid of fetal lamb at a time in gestation when morphologic

and biochemical evidence indicates that pulmonary surfactant is being secreted, and the secretion of apoproteins into tracheal fluid of fetal lamb can be stimulated precociously by the administration of dexamethasone. The glucocorticoid activity of dexamethasone has been shown to accelerate the synthesis and secretion of pulmonary surfactant(76). Apoproteins have been found in human amniotic fluid at gestational ages in which biochemical and clinical evidence indicates that pulmonary surfactant is present. Change in the concentration of apoproteins in amniotic fluid with increasing gestational age is paralleled by the change in the concentration of surface-active phospholipids, indicating the secretion of pulmonary surfactant into amniotic fluid; and suggests, again, that surfactant may be secreted as a lipoprotein during fetal development (44).

At present the characterization of surfactant components is far from complete. The material is complex and a precise stoichiometry among its components has not yet been demonstrated. Its composition may vary among animal species, with age of the individual, during its passage through the lung, and possibly even with the individual's physiologic state. Surfactant is probably only one of many substances secreted onto the alveolar surfaces. Very few studies, to date, have been done on the physiologic roles of the glycoproteins in lung surfactant, and their metabolic

relationship to the lipid of surfactant is still unknown. What about the order of amino acids in these glycoproteins, and the nature and position of the carbohydrate groups in these peptides? Such sequences must determine the configuration of at least the peptides and may influence the arrangement of other components in, and the functional properties of the complex as a whole. Do the apoproteins of surfactant organize the structure of the surfactant, intracellularly, or in the alveolar extracellular space?

What factors influence the secretory process? Physiologic studies suggest that respiratory frequency may be a determinant of the rate at which surfactant is utilized on the alveolar surface (22, 58). Since replacement should keep pace with utilization, respiratory rate might be a determinant of secretion as well as utilization. Examination of the appearance of radioactive protein in surfactant returns reveals that a greater amount appears per unit time in the lavage of species with high compared to those with low respiratory rates (57). There may be two replacement mechanisms for the ventilatory-induced depletion of surfactant in the surface film of the duplex alveolar extracellular lining layer. Firstly, a greater rate of adsorption might occur in small animals of the surface-active material into the surface film from the hypophase of the duplex lining layer. These animals

have a higher respiratory breath by breath replacement of surfactant. This might be effected by a greater amount of protein in the hypophase which, *in vitro* seems to accelerate adsorption of surface-active material (42). A greater per cent protein occurs per unit time in lavage fractions from species with high respiratory rates. The second replacement mechanism would be the secretion of surface-active material (also related to respiratory rate) into the hypophase from the lamellar body storage granules. This would probably be not as fine a control as the first mechanism.

IV. PURPOSE OF STUDY

In accordance with the present interest in the function and biosynthesis of these surfactant glycoproteins, this study was undertaken to characterize and study the properties of glycosyltransferases in the lung microsomes, larvage surfactant and lamellar bodies. It was anticipated that study of some of these enzymes would provide some clues to the current, much sought after answers concerning their role in surfactant, and, in lung in general. Glycosyltransferases have previously been found in the mucosal lining of canine respiratory tracheas, as part of the tracheo-bronchial mucus secretions. These enzymes transfer fucose, GalNAc, GlcNAc, galactose and sialic acid to their respective acceptors (6). These secretions may contain components of alveolar

secretion that have spread from the alveolar surface to the surface of conducting airways. UDP-galactose glycoprotein galactosyltransferase activity has also previously been found to be high in fetal lung. This activity was found to decrease with gestational age and with no further changes after birth (38). Changes in the secretion of lung surfactant probably contribute significantly to the disease state and may be attributable to abnormal properties of glycoproteins (structural changes). They should be a reflection of change in the activity of the enzymes involved in their biosynthesis or alterations in the physiological control mechanisms regulating their biosynthesis.

MATERIALS AND METHODS

I. MATERIALS

A. Animals

Male adult Sprague-Dawley rats, weighing 300-400gm used in all experiments were obtained from Canadian Breeding Farms and Laboratories, St. Constance La Prairie, Quebec. They were kept under continuous light conditions with free access to Purina Rat Chow (Ralston Purina of Canada, Ltd., Don Mills, Ontario) and drinking water. After receipt of shipped animals, they were conditioned in animal facilities for at least one week prior to use. In some experiments, rabbits (weighing 6-8 lbs.), were used for the study of the properties of lung lamellar bodies and lung lavage. The procedures used were exactly similar to those that utilized rats.

B. Reagents

All chemicals were of commercial origin, purchased from Fisher Scientific Co., Halifax, Nova Scotia, and Serdary Research Laboratories (London, Ontario). All organic solvents were of analytical grade.

C. Radioisotopes

Radioactive substrates UDP-(U-^{14}C) galactose (specific activity 0.301mCi:/mmol) and CMP-(4-^{14}C) sialic acid (specific activity 1.68 mCi:/mmol) were purchased from New England Nuclear Corp., (Dorval, Quebec).

D. Source of Materials

Lecithin (egg yolk), Lysolecithin (egg yolk) sphingo-

myelin, cholesterol, ganglioside, ovalbumin, Triton X-100 and fetal calf serum fetuin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Lysolecithin, (pig liver) and (oleoyl - synthetic), Lyso PE, lyso PA (egg) and (synthetic) and all other lipids used were obtained from Serdary Research Laboratories (London, Ontario). All lipid preparations gave a single spot on TLC. The molecular weight of 536 for lysolecithin was arbitrarily calculated (as oleoyl lysolecithin) (94), and the molecular weight of Triton X-100 was calculated as 635 on the basis of a structure of polyoxyethylated octylphenol (ethylene oxide : 9.7 units) (94) Acrylamide, bisacrylamide, ammonium persulphate, N,N,N',N' — tetramethylene diamide (TEMED) and pure sodium dodecylsulphate, which were used for polyacrylamide gel electrophoresis, were purchased from Sigma Chemical Co., Human α_1-acid glycoprotein used in the preparation of some of the glycoprotein acceptors was a gift from the American Red Cross National Fractionation Centre, Washington, D.C., U.S.A.

II. ACCEPTOR PREPARATION

A. Preparation of DS- and DSG- fetuin

Fetuin contains galactose in two different oligosaccharide chains: one containing sialic acid, galactose, mannose and N-acetylglucosamine, joined to the peptide through an N-acetyl-glucosamine-asparagine linkage that is stable to mild alkali treatment: the second, and minor one, contains sialic acid, galactose, and N-acetylgalactosamine joined through the alkali-labile linkage N-acetyl galactosamine — serine or —threonine.

Sialic acid was removed from fetal calf serum fetuin by mild acid hydrolysis (93). Sequential degradation of the monosaccharides of fetuin was performed by periodate oxidation followed by reduction with $NaBH_4$ and mild acid hydrolysis as described by Spiro (93). When this method is used, only hexosamines and mannose remain attached to the peptide portion of fetuin.

B. Preparation of DS - and DSG - α_1 ,acid glycoprotein

Sialic acid free α_1 — acid glycoprotein used as acceptor for sialyltransferase assay was prepared by mild acid hydrolysis (93). Sialic acid and galactose depleted α_1 — acid glycoprotein used as acceptor for galactosyltransferase assay was prepared by the periodate oxidation and borohydride reduction method (93).

C. In some experiments, native ovalbumin, a glycoprotein whose carbohydrate moiety contains only N-acetylglucosamine and mannose was used as acceptor for galactosyltransferase.

III. PREPARATION OF EXPERIMENTAL TISSUE

A. Dissection

Rats were anaesthetized with intraperitoneal injection of sodium pentobarbitol (60mg/lb. live weight) and the lungs were then perfused *in situ* via the inferior vena cava with cold 0.9% saline. The lungs were then immediately excised. All subsequent operations were done at 4° C. The tissue was blotted dry, weighed, finely minced with scissors and then homogenized in two volumes of TKM buffer (50 mM Tris-HCl, pH 7.5, containing 0.025 M KCl and 5mM $MgCl_2$) in 0.25 M sucrose, using a Potter-Elvehjem motor-driven homogenizer with a loose fitting teflon pestle (clearance 0.1-0.2 millimeters). The resulting homogenate was filtered through two layers of cheesecloth.

B. Preparation of Crude Microsomes

Figure 4 shows the preparation of crude microsomes from lung homogenate. The homogenate was centrifuged at 10,000 r.p.m. for 10 minutes in a Sorvall RC-5 centrifuge in order to sediment the cell debris, nuclei, and mitochondria. The post-mitochondrial supernatant was centrifuged at 100,000 xg (38,000 r.p.m.) for one hour in a Beckman ultracentrifuge (model L-50) to obtain the total microsomal fraction as a pellet.

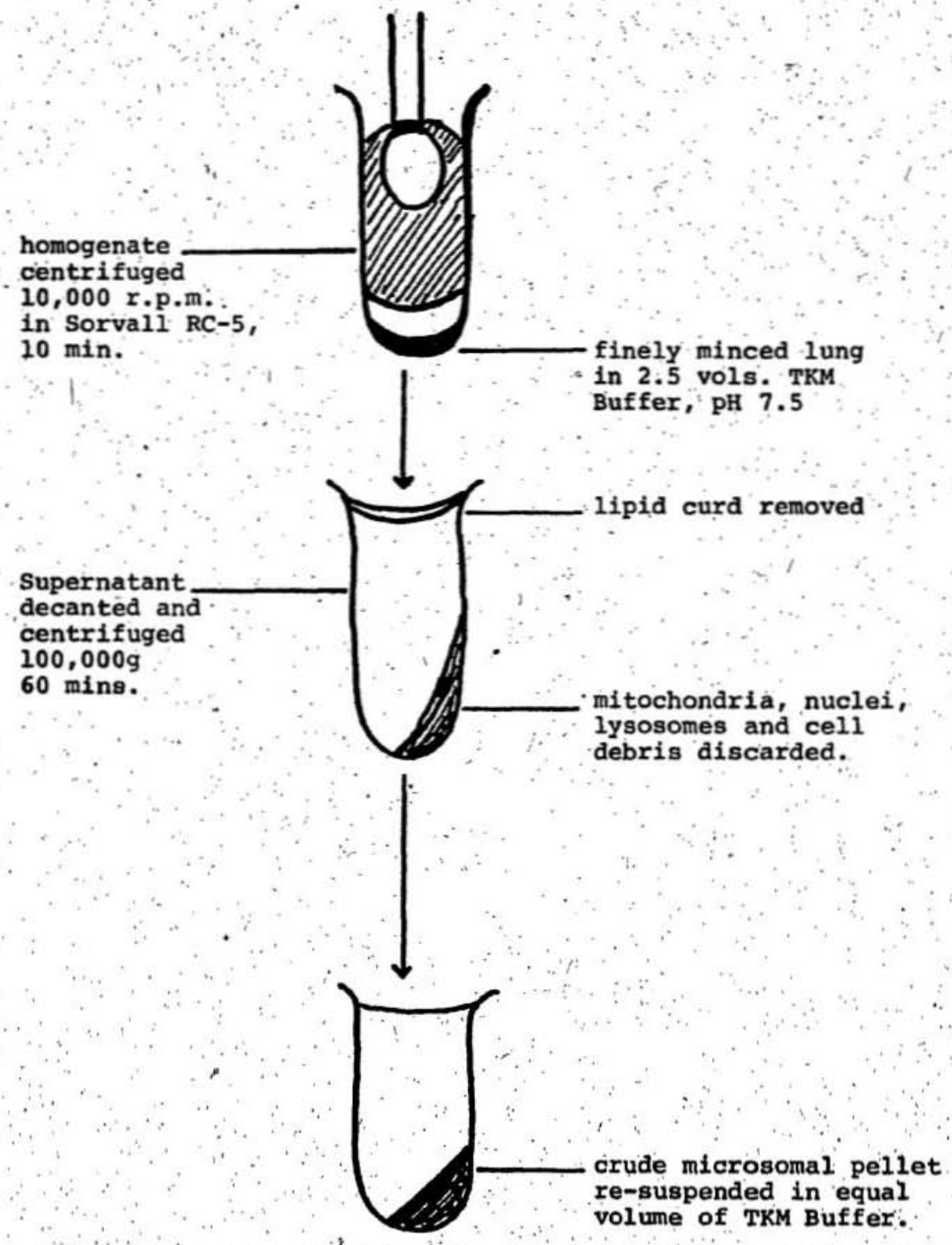


FIGURE 4 - PREPARATION OF CRUDE MICROSOMES (61)

The microsomal pellet was suspended in TKM buffer 1:1 (v/v) and then either used fresh for the assay of sialyl-and galactosyltransferase, or else stored at -20° C (in 0.5 ml aliquots to prevent repeated thawing and freezing of the samples).

C. Preparation of Surfactant

Surfactant particulate material was prepared from lung lavage according to the method of Bhattacharyya et al (8). Fifteen to twenty rats were required in each experiment to obtain approximately 50 mg of lavage protein. The rats, under sodium pentobarbital anaesthesia, underwent in situ lung perfusion via the inferior vena cava as described before. A 10 c.c. syringe with a blunt needle was then inserted into the trachea. Cold 1mM hypotonic EDTA in 5mM Tris-HCl buffer, pH 7.5, was slowly injected and the lungs gently lavaged to and fro. Ten to fifteen mls lavage material was collected from each rat. The hypotonicity of the lavage solution was found to be necessary in order to increase the amount of protein and particulate material in the washings. An isotonic saline solution removes all the lipid but very little protein is obtained if this is used for lavage washings. Figure 5 illustrates the procedure for isolating surfactant particulate material from starting lung lavage. Lavage was centrifuged at 750g for 10 minutes to pellet down alveolar cells and other broken cells removed by lung lavage. The supernatant was centrifuged at 25,000g 15 minutes and the resulting precipitate re-suspended in buffer and gently homogenized. This homogenate

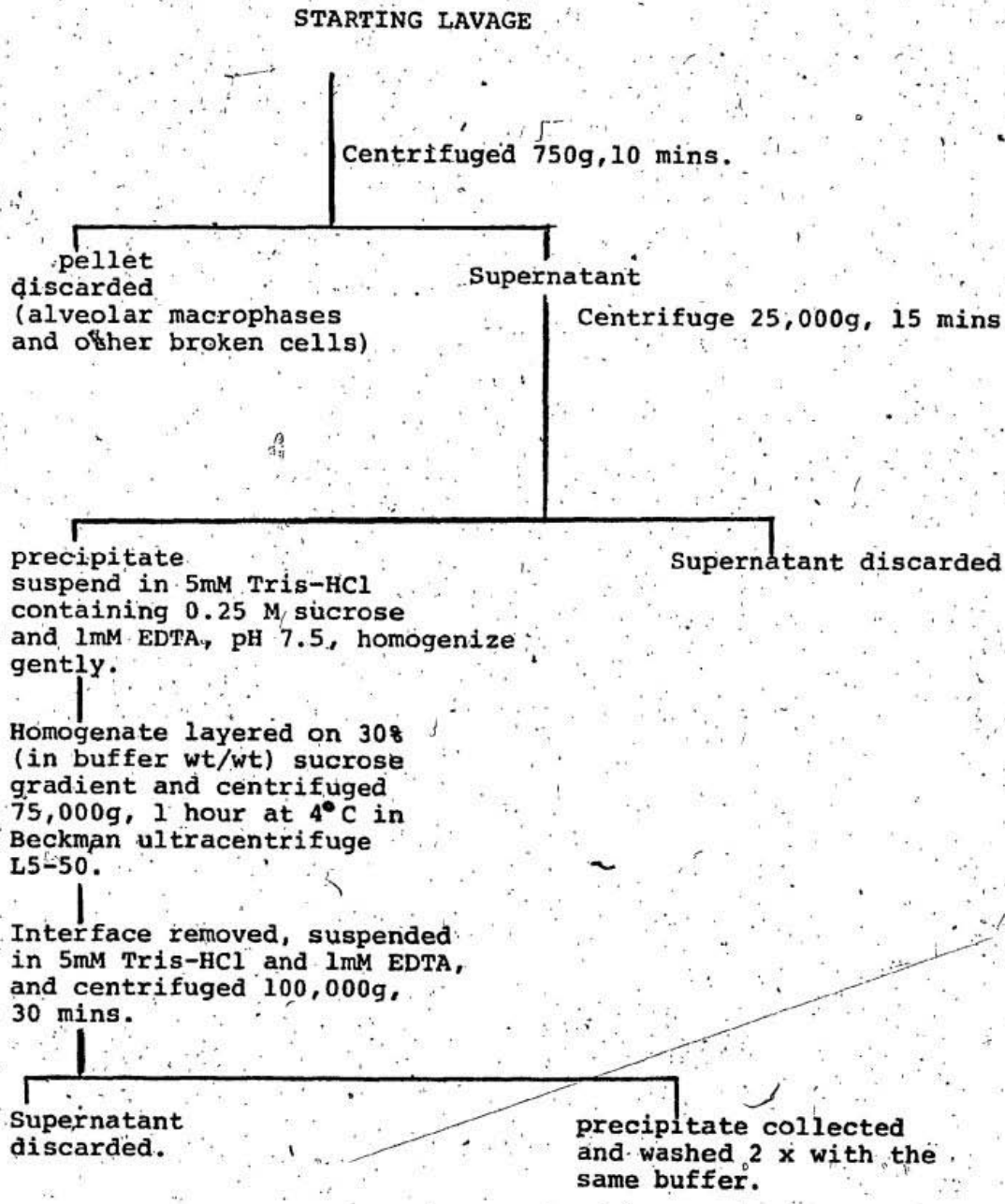


FIGURE 5. ISOLATION OF SURFACTANT MATERIAL FROM RAT LUNG LAVAGE.

was layered on a 30% sucrose solution and the gradient then spun for one hour at 75,000g in the ultracentrifuge. The surfactant material was localized in the interface that resulted. This interface suspended in the same buffer, was centrifuged 100,000g for thirty minutes. The precipitate collected was washed twice with this buffer in order to remove sucrose and other soluble proteins. All preparatory fractions were saved for glycosyltransferase assay.

D. Preparation of Lung Lamellar Bodies

The methods used by most investigators are modifications of those of de Duve and co-workers (16,17) and utilize differential and density gradient centrifugations after a comparatively gentle homogenization. Lamellar bodies were isolated from rat lung in this study according to the method of Di Augustine (18). Rats, in groups of 10-20, were used and the lungs excised in the same manner as for microsome and lavage preparation, except that lung was suspended in two volumes of 0.25M sucrose - 0.05M Tris-HCl (pH 7.2 - 7.3) containing 0.1 mM EGTA.

This method for lamellar body isolation was preparative, rather than analytic, for the sake of purification of these organelles. Therefore, because volumes are discarded in the various fractions, total enzyme activity yield in each fraction was compromised for purity. Figure 6 shows the method of isolation of the lamellar bodies from starting lung homogenate.

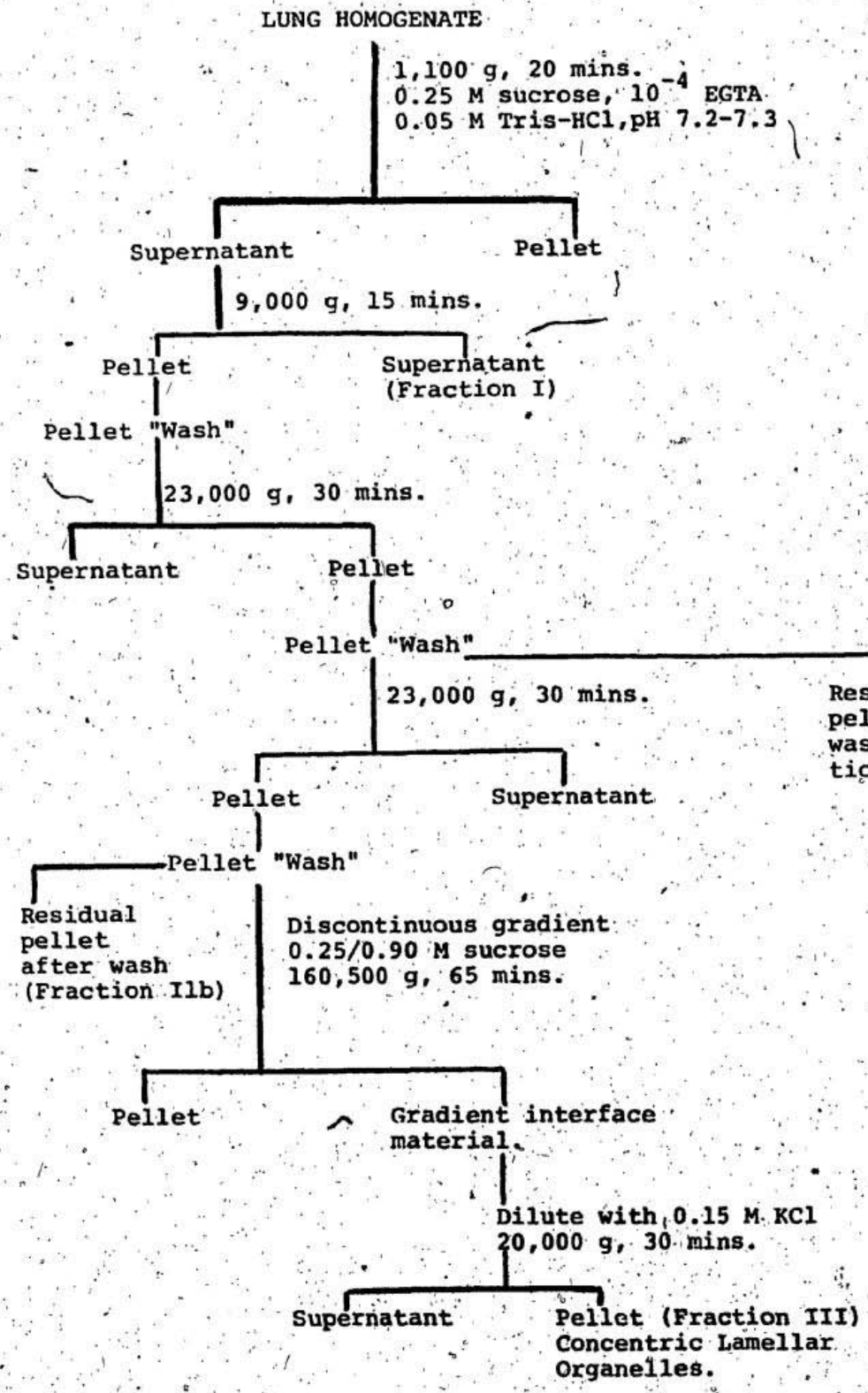


FIGURE 6. FLOW DIAGRAM FOR THE PREPARATION OF RAT LUNG CONCENTRIC LAMINAR ORGANELLES.

Rat lung homogenate was spun at 1,100 g for 20 minutes in a Sorvall RC-5 centrifuge in order to pellet down the nuclear fraction. The supernatant layer and only that devoid of loose pellet, was then spun at 9,000 g for 15 minutes, after which the supernatant containing the microsome fraction (fraction I) was discarded. The remaining pellet was carefully overlaid with 0.5 - 1.0 ml of Tris-HCl buffer and then gently rocked to and fro over the pellet in order to wash out the lamellar bodies from the mitochondrial fraction. This was repeated until washings became clear. This total pellet wash was then centrifuged at 23,000g for 30 minutes, after which the resulting pellet was again subjected to a similar wash. This wash was centrifuged as before, at 23,000g for 30 minutes. The third pellet wash was then applied to a discontinuous gradient, 0.25 wash/0.90M sucrose and spun at 160,500g for 65 minutes, using a 60 Ti rotor in a Beckman ultracentrifuge, model L5-50. Upon completion of the run the gradient interface material was collected with a pasteur pipette and then further diluted with 0.15M KCl. This material was centrifuged at 20,000g for 30 minutes. This wash was necessary in order to remove any sucrose remaining. The pellet (Fraction III) containing the lamellar bodies was collected, re-suspended in 0.05M Tris-HCl buffer and frozen at -20° C. A small portion of all fractions obtained during the isolation procedure was saved for later assay of glycosyltransferases.

IV ASSAY METHODS

A. General

Glycosyltransferases (sialyl-and galactosyl-) were measured in lung homogenates(75 µg protein/assay), microsomes (85-95 µg protein/assay), lung lavage (5-6 µg protein/assay) purified lamellar bodies (1.0 µg/assay) and in all other intermediate fractions obtained in the isolation procedures. (1.5 - 170 µg protein). The enzyme assays were proportional with the time of incubation and with enzyme protein concentrations used. Enzyme activities were found to be reproducible within 15% when assays were repeated under the same conditions. Unless otherwise specified, the following assay conditions were used:

B. Sialyltransferase

-MES buffer, pH 6.8, 6.25 µmol, in 5 µl.

-Triton X-100, 0.3% (V/V) final concentration 5 µl.

-Desialized fetuin acceptor, 250 µg in 5µl.

-CMP- $[4-^{14}C]$ sialic acid, 6.3 nmol/0.01 µCi/20,000 c.p.m. in 5 µl.

-dH_2O, 15 µl, or amount required to adjust the final volume.

-Enzyme preparation 15 µl.

Total assay volume of 50 µl.

C. Galactosyltransferase

-MES buffer, pH 6.8, 6.25 µmol, in 5 µl

-Triton X-100, 0.75% (V/V), 5 µl

-$MnCl_2$, o.625 µmol, 5 µl.

-ATP, 2mM, pH 6.8 - 7.0, 5 µl (ATP was included in this assay to prevent UDP-galactose pyrophosphatase activity (65).

-Desialized and degalactosylated fetuin acceptor 250 µg in 5 µl

-UDP - galactose ^{14}C: 3nmol/0.025 µCi/49,500 c.p.m. in 5 µl.

-dH_2O, 5 µl, or amount required to adjust the final volume.

-Enzyme preparation, 15 µl.

Total assay volume 50 µl.

D. Assay Procedure and Determination of Enzyme Activity.

Incubations of all assay ingredients were carried out in small culture tubes in a shaking water bath maintained at a constant 37° C for 60 minutes, The reaction was then stopped with 1ml of TCA (10% W/V)/PTA (2% W/V) in a 1:1 solution and the assay tubes vortexed and placed on ice. The precipitate was filtered under suction through glass fiber filters (Whatman GF/A) and washed with a large excess of cold TCA-PTA (5%/1%) containing 0.5% galactose followed by 7 mls ethanol/ether (1:1 V/V) and then 5 mls ether. The paper discs were then dried and transferred to scintillation vials. Twelve mls of a toluene-based scintillation fluid containing 3.92 gms of PPO (2,5-diphenyloxazole) and 80 mg of POPOP [1,4 bis-(5-phenyl-oxayolyl) benzene] /litre. The incorporation of radio-activity into exogenous acceptor protein was measured in a Beckman LS 9000 model liquid scintillation counter and was recorded as counts per minute (c.p.m.). This unit of measure-ment was then used in the calculation of enzyme specific activity

(nmol sugar C^{14} incorporated/mg protein/hour) by standard procedures.

To determine the endogenous activities of both enzymes, the respective exogenous glycoprotein acceptor was excluded from the reaction mixture and a small % (2-8) of counts due to endogenous activity was deducted for obtaining the results. Assays for glycosyltransferases utilizing endogenous acceptors can give misleading results:

(1) tissues frequently contain several glycosyltransferases that utilize the same sugar nucleotide but different acceptors, and incorporation into endogenous acceptors does not define which enzyme is being measured,

(2) the endogenous acceptor, not the enzyme, may be the rate limiting component of such incubation mixtures. Also, the enzyme activities with endogenous acceptors are low and difficult to measure accurately.

E. Assay of Sialyl- and Galactosyltransferases in the Presence of Varying Amounts of Lipids and Detergents.

When the effect of exogenously added lipids on the assays of sialyl- and galactosyltransferase were studied, they were added in chloroform – methanol (2:1, V/V) to an empty culture tube and taken to dryness under nitrogen. The microsome preparations were then added and the lipids solubilized by repeated vortexing prior to the addition of the other assay ingredients. Assays for the glycosyltransferases were the same as outlined in Section IV B & C, except that all individual

volumes were doubled, bringing the total volume of assay to 100 µl.

If Triton X-100 was omitted from these assays, the volume was made up with dH_2O. When Triton X-100 was to be included in the assays containing lipids, it was first solubilized and added with the dried lipids by vortexing, after which the microsome preparation was added with further vortexing, followed by addition of other assay ingredients, as before. Further assay procedure was as described in IV D.

F. pH Optima Studies

In the assays used to test optimum pH requirements (Fig.10 A & B), the four different buffers studied were first added to all assay ingredients, except for the nucleotide-sugar-donor and the enzyme, and the various pH's were checked for consistency after mixing. The pH values plotted are those of the final assay solution (minus radioactive substrate) rather than the pH of the stock buffer solutions alone. All assay ingredients were increased 5-fold when the pH profiles were determined.

G. Subcellular Marker Enzyme Assays

The following enzyme assays were used to characterize the subcellular fractions (Lh, Fractions I,II and III as shown in Figure 6) obtained during the purification of lung lamellar bodies:

(i) Acid Phosphatase was assayed according to the method of Schachter *et al* (83) as a marker for lamellar bodies.

(ii) Succinate cytochrome C reductase, a marker for

mitochondria and NADPH-cytochrome C reductase (microsomes) were assayed according to Sottocasa et al (92).

(iii) Lactate Dehydrogenase, a cytoplasmic enzyme marker was assayed according to Morrison et al (71).

H. Protein Determination

The protein in all tissue fractions used for enzyme assay was determined by the method of Lowry et al (51), using bovine serum albumin as a standard.

V. THIN LAYER CHROMATOGRAPHY

All lipids used in assay studies with galactosyl-transferase and sialyltransferase were tested for purity on TLC plates. The plates were coated according to the manufacturer's instructions using the "Quickfit" apparatus for spreading the silica gel absorbent slurry. These plates were activated by heating to 110°C for one hour before use.

(i) Spotting - For single lipid standards, 25-50 µl (containing 100-200 µg lipid) proved the best volume to use. For phospholipid standard mixtures, up to 100 µl were used. Development was by ascending chromatography in glass tanks.

ii) Solvent Systems

Phospholipids

Chloroform/methanol/water (65:25:4/V:V)

Neutral Lipids (triglycerides)

n - hexane/diethyl ether/glacial acetic acid/methanol (90:20:2:3/V:V)

(iii) Chemical Visualization of Thin Layer Chromatograms

plates were sprayed with concentrated sulphuric acid (+ Na nitrate)

Every organic material on the chromatogram is then charred by heating the plate in an oven at 100°C (black spots on white background).

VI. ANALYTICAL POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gels (7%, V/V) were prepared in 0.375 M - Tris-HCl buffer, pH 8.3 for 180 minutes at a current of 2mA/gel, according to the method of Davis (15).

For molecular-weight determination, SDS/polyacrylamide gel electrophoresis was carried out as described by Weber and Osborn (100). Molecular weight estimations for peptide on gels were made with cytochrome C, fetuin, chymotrypsinogen, myoglobin, bovine serum albumin and ovalbumin as standards. Analytic gels were stained first with Coomassie blue as a guide to determine an approximate mobility range for the glycoprotein band. A modified periodic acid/Schiff technique was used to stain for carbohydrates in the glycoproteins, as described by Glossman and Neville (28).

VII. REPRODUCIBILITY OF DATA

All experiments reported in this thesis were repeated three or four times in duplicate and the results were reproducible.

RESULTS

1. STUDIES WITH LUNG HOMOGENATE

Initially, sialyl and galactosyltransferase activities were measured in the total lung homogenate to ascertain the extent of their activity in lung tissue. In the past, the study of glycosyltransferases has been confined mainly to the upper trachea and the mucosal secretions of the lung, with little or no study on whole lung tissue or its sub - cellular fractions. Figure 7 shows the rate of these two enzymes activities measured as a function of the amount of lung homogenate protein. Both enzymes are highly active in lung tissue.

Examination of various properties of the enzymes indicated a differential requirement for the detergent Triton X-100. Detergents are used in the assay of glycosyl - transferases in membrane material, especially with intact organelles of closed vesicles, where the enzyme may be located on the outer or inner face of the menbrane. The detergents, through a solubilizing effect (micellar formation), ensure that the substrate is presented homogeneously to the activity site. Although the inclusion of mild detergents in *in vitro* glycoprotein studies has been found to optimize the tissue glycosyltransferase activity, Triton X-100 is certainly not a prerequisite *in vivo* where this detergent is not found. The effect of Triton X-100 dosage on

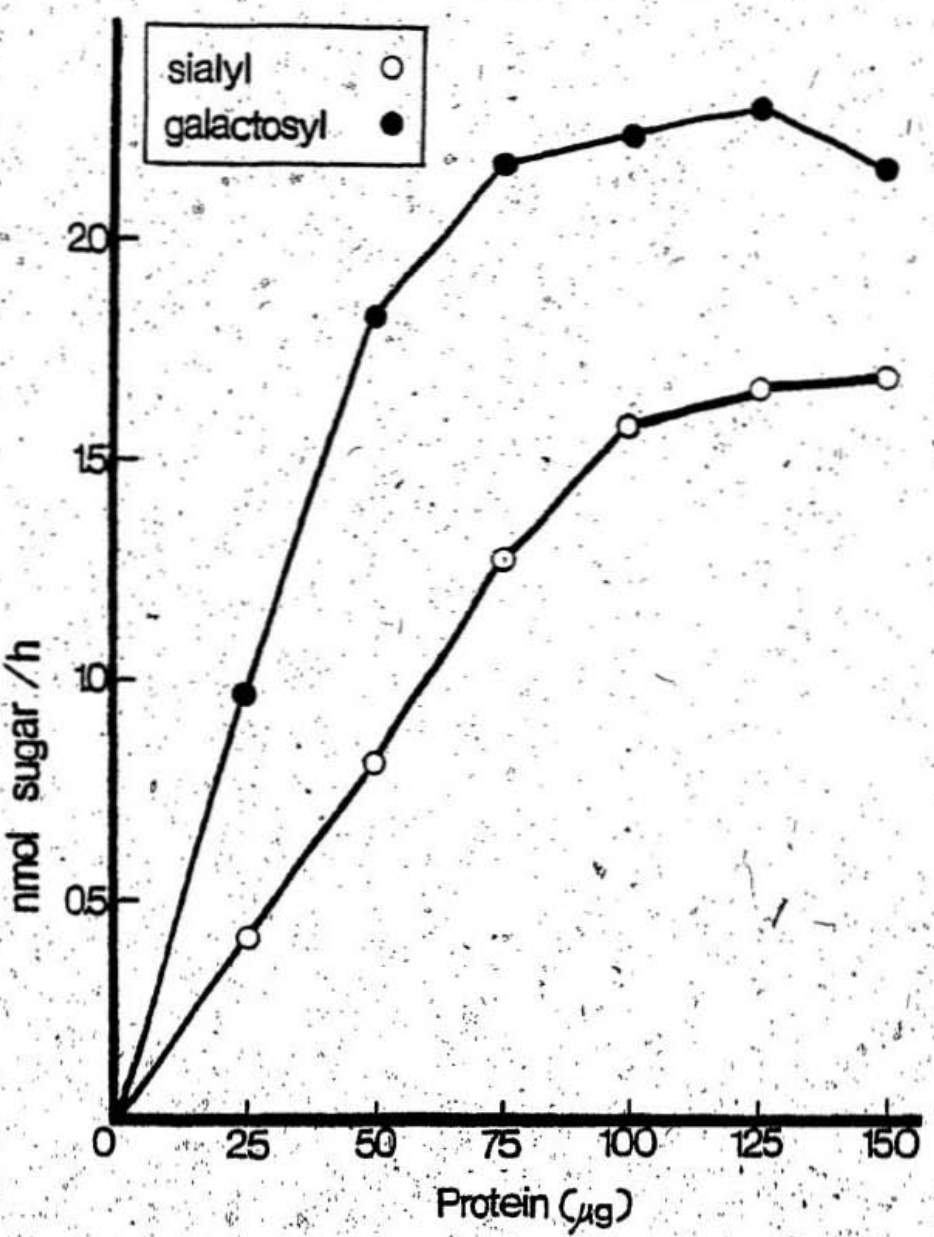


FIGURE 7. Glycosyltransferase Activity as a Function of Lung Homogenate Protein.

the enzymes' activities in lung homogenate is illustrated in Figure 8. The results suggested that, although galactosyltransferase activity was dose-dependent up to 0.75% Triton, sialyltransferase was unaffected by large increments of the detergent. The former enzyme's activity was highest at 0.75% Triton (final assay dose) while the latter enzyme was most active using 0.3%.

In these initial trial experiments the specific activity of sialyltransferase was increased two-fold in rat lung microsomes over that of the total homogenate, where as galactosyltransferase activity rose six-fold in the microsomes (Table 2). Again, for microsomal galactosyltransferase, as with lung homogenate, a large difference in activity was observed when Triton was either included or omitted from the assay (for microsomes, an eight-fold difference in the specific activity). Sialyltransferase activity in the microsomes showed only a maximum two-fold increase when the optimum 0.3% Triton was included in the assay (Table 2).

Table 2 also gives the specific activities found in the post-microsomal supernatant, for the two glycosyl - transferases. A proportionally higher sialyltransferase activity was found in this fraction as compared to the galactosyltransferase. This explains, in part, why sialyltransferase activity rose only two-fold when microsomes were compared to total lung homogenate, and also why Triton X-100 had little effect on this enzyme. Most intra-

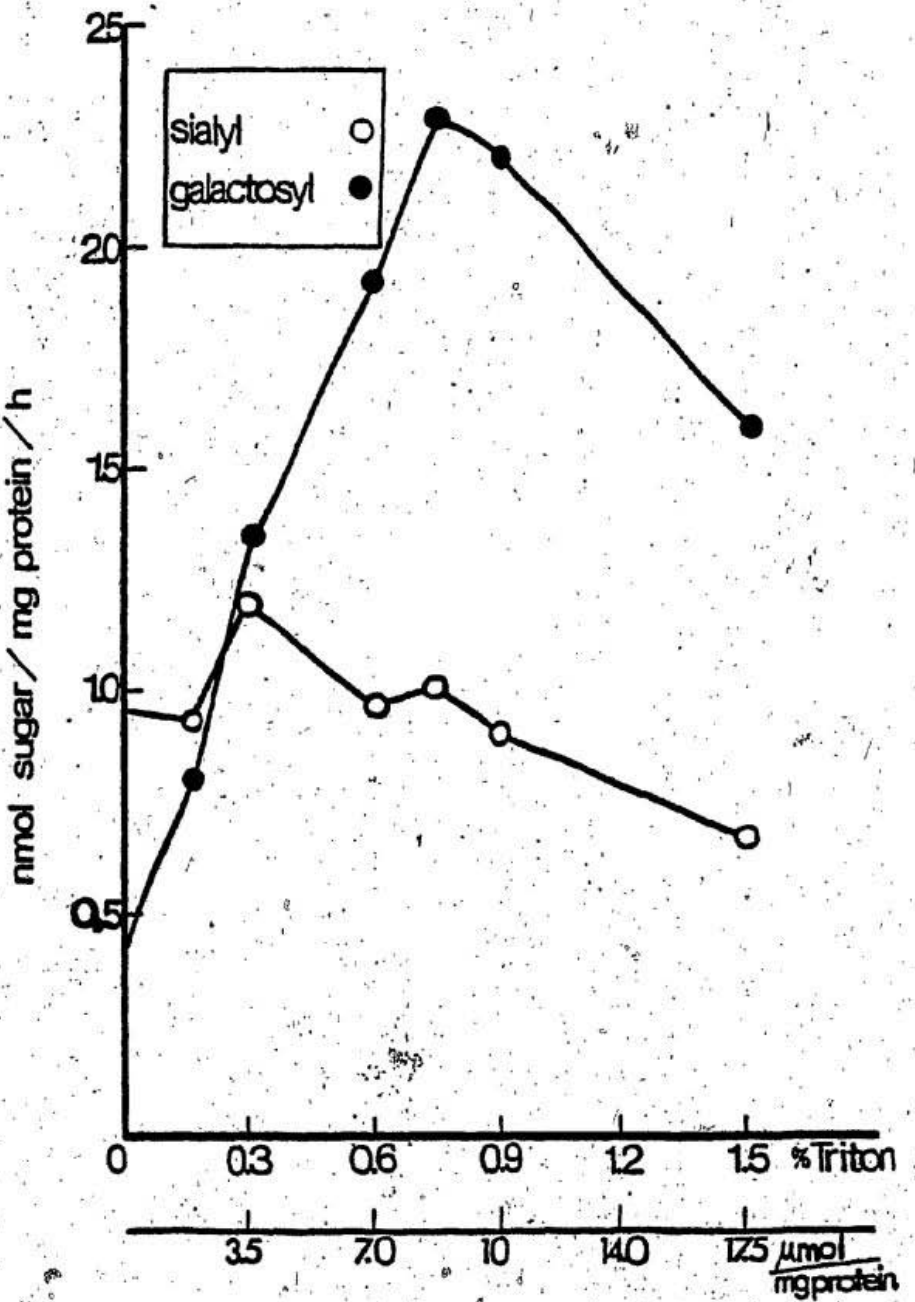


FIGURE 8. Glycosyltransferase Activity in Lung Homogenate as a Function of Triton X-100 Dose.

TABLE 2

EFFECT OF TRITON X-100 ON GLYCOSYLTRANSFERASE ACTIVITIES IN RAT LUNG TISSUE FRACTIONS. *

SPECIFIC ACTIVITIES

	Triton Addition	Lung Homogenate	Micro-somes	Post Micro-somal • Super-natant.
SIALYLTRANSFERASE	+	1.26	2.26	0.74
	−	0.95	1.21	0.51
GALACTOSYLTRANSFERASE	+	2.30	14.02	0.60
	−	0.45	1.75	0.30

Results as reported here represent the average of duplicate analyses from three separate experiments (10 rats/experiment).

* Results are expressed as nmol/mg protein/h

x Optimum Triton dose (sialyl-0.3%, galactosyl-0.75%) used when included in assay.

• Supernatant obtained after 100,000 g final microsome centrifugation

cellular glysosyltranferases are bound with varying degrees of tenacity to the menbrane system of the cell. A difference between the two glycosyltransferases in this respect was indicated in these initial experiments.

II. PROPERTIES OF SIALYL- AND GALACTOSYLTRANSFERASE ACTIVITY IN LUNG MICROSOMES.

Rat lung microsomes were used to study the properties of these two glycosyltransferases in more detail.

A. TIME AND PROTEIN DEPENDENCE

The dependance of sialyl- and galactosyltransferases activity on protein concentration and incubation time are illustrated in Figure 9, A-D. Microsomal sialyltransferase activity was proportional up to about 90-100 µg of protein (Fig. 9C). Similar proportionality was observed for galactosyltransferase (Fig. 9D). The activities of both enzymes were linear during a sixty minute assay incubation time (Fig. 9 A,B). In subsequent glycosyltransferase assays a 15 µl microsome suspension (80-90 µg protein) was employed for sixty minutes to maintain the optimum rate of activity.

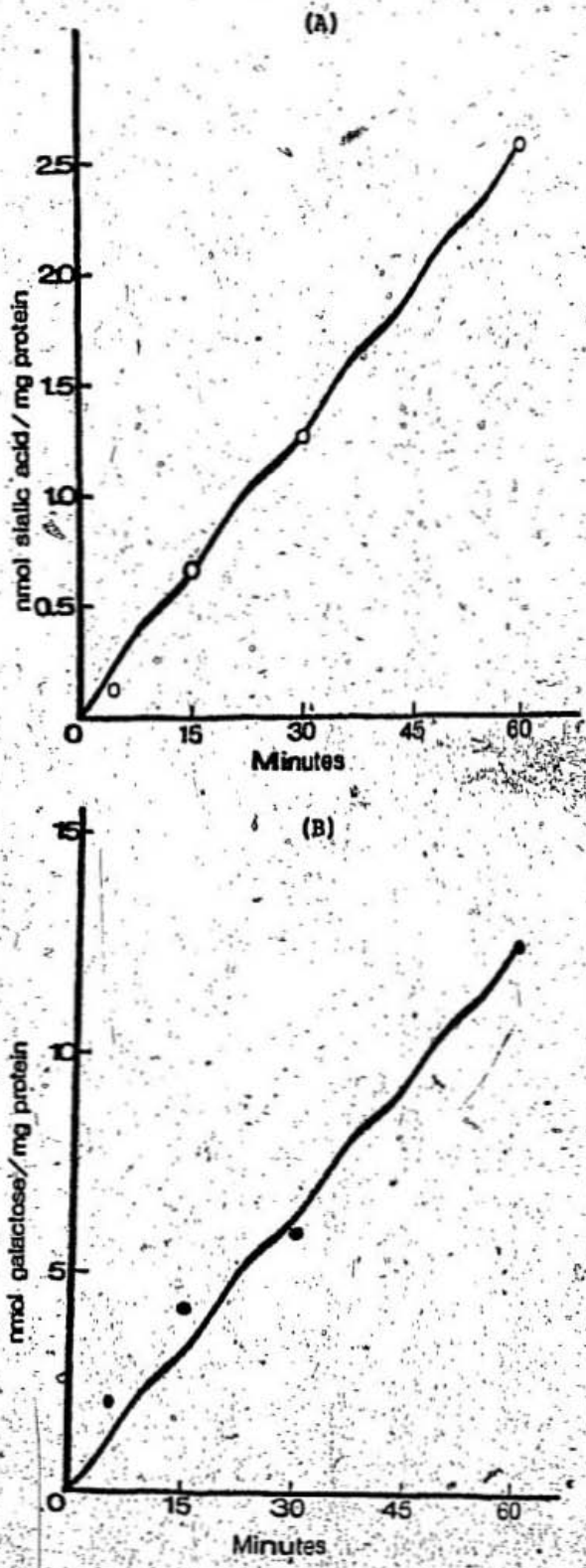
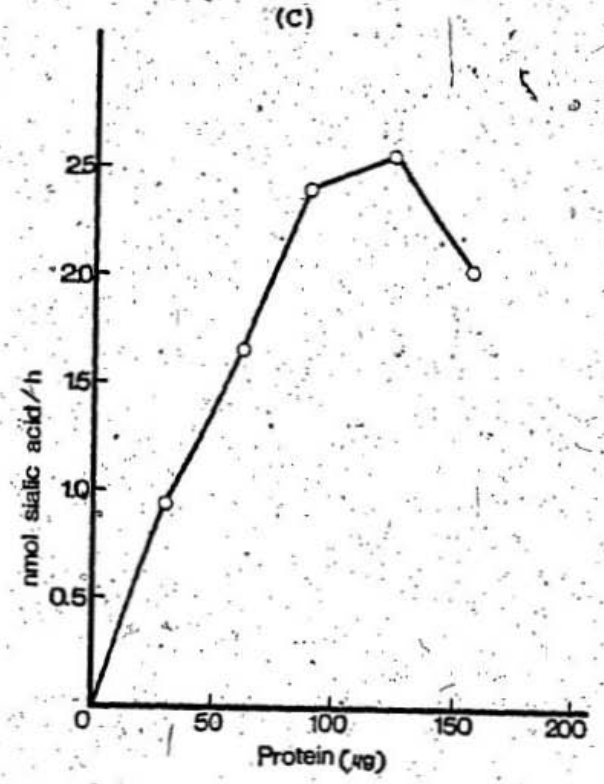


Figure 9. Glycosyltransferase Activity in Lung Microsomes as a Function in Incubation Time (A,B) and Enzyme Protein (C,D).



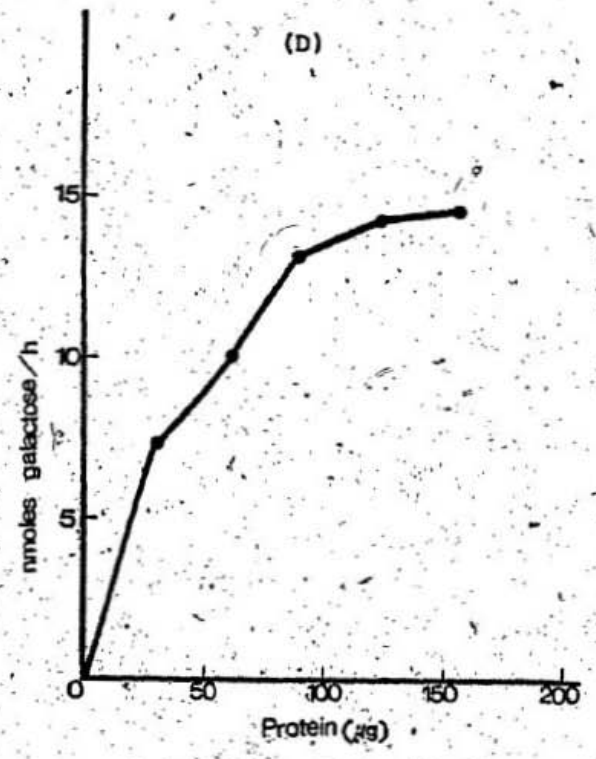


Figure 9. Glycosyltransferase Activity in Lung Microsomes as a Function of Incubation Time (A,B) and Enzyme Protein (C,D).

The requirements for Triton, ATP, and MES buffer, are shown in table 3, section A, using the complete assay system as described in Materials and Methods. Section B gives the relative effect of using different exogenous acceptors and Section C shows the effect of varying the sugar-donor substrate concentration on each enzyme, in the presence and the absence of Triton X-100. Section D gives the cation requirements for galactosyltransferase.

B. NUCLEOTIDE REQUIREMENTS

Cellular levels of UDP-sugar donor substrates are known to control their own biosynthesis by a negative feedback mechanism. As well, liver microsomes have shown very active uridine diphosphate-galactose pyrophosphatase activity leading to the hydrolysis of uridine disphosphate-galactose into galactose-1-phosphate, and finally into galactose. This provides an alternate means of preventing the excessive accumulation of nucleotides in the cell but can also affect the kinetic properties of the membrane-bound galactosyltransferase. Both glycosyltransferase and nucleotide-sugar pyrophosphatase enzymes are present in the cytomembranes and therefore, a concerted action of both on nucleotide-sugar substrates may exert a control on protein glycosylation reactions. A number of nucleotides and their derivatives, ie., CTP, ATP, GTP, and CDP-choline have been found to inhibit the action of pyrophosphatase, and in past studies using liver tissue (65)

TABLE 3

PROPERTIES OF GLYCOSYLTRANSFERASES IN RAT LUNG MICROSOMES

SIALYLTRANSFERASE (nmol/mg protein /h)		GALACTOSYLTRANSFERASE (nmol/mg protein/h)	
A. Requirements		A. Requirements	
Complete *	2.4*	Complete	14.0
- Acceptor	0.4	- Acceptor	0.4
- Triton X-100	1.3	- Triton X-100	1.75
- MES	1.1	- MES	9.50
		- ATP	7.41
B. Protein Acceptors +		B. Protein Acceptors	
- Acceptor	0.4	- Acceptor	0.4
+ DS — fetuin	2.5	+ DSG — fetuin	13.2
+ DS α_1-acid glycoprotein	0.82	+ DSG α_1-acid glycoprotein	2.9
+DS-Mucin (submax)	0.7	+ Ovalbumin	11.0
+DS-Mucin (stom.)	0.28		
C. Effect of CMP-sialic acid		C. Effect of UDP -galactose	
Apparent Km (+Triton)	0.33mM	Apparent Km (+Triton)	0.053mM
(-Triton)	0.5 mM	(-Triton)	0.067mM
Vmax (+Triton)	10	Vmax (+Triton)	2.0
(-Triton)	6.66	(-Triton)	0.33
		D. Cation Requirements	
		+ Mn^{2+}	13.5
		Mn^{2+}	1.5
		- Mn^{2+} + Ca^{2+}	1.0
		- Mn^{2+} + Mg^{2+}	0.5
		- Mn^{2+} + Co^{2+}	4.5

* Complete assay system used as described in Materials & Methods

+ All exogenous protein acceptor values are recorded at 250 µg dose

the addition of nucleotide ATP was necessary for maximum stimulation of galactosyltransferase.

Sialyltransferase activity was unchanged whether or not ATP was included. Omission of ATP from galactosyltransferase assay, however, resulted in 50% decrease in enzyme activity (Table 3A), indicating the presence of UDP-galactose nucleotide pyrophosphatase in rat lung microsomes. The stimulatory effect of ATP on galactosyltransferase is partly related to its inhibitory effect on the pyrophosphatase, due to increased substrate protection.

C. OPTIMUM pH FOR THE ASSAY

Enzyme activities were studied as a function of different buffers with different pH. The assay technique is described in Materials and Methods. In Figure 10A sialyltransferase is most clearly active with MES buffer at a pH of 6.8. Galactosyltransferase (Fig. 10B) showed a much broader pH and buffer requirement for optimum activity, although, as with the former enzyme, MES buffer, pH 6.8 gave the maximum effect. This less specific requirement could also account for the fact that omission of MES from the assay caused a smaller decrease in the galactosyl - transferase activity than with sialyltransferase (Table 3A). MES buffer, pH 6.8 was used in all subsequent assays for both enzymes.

LEGEND FOR FIGURE 10 (A,B)

Buffers (6.25μmol) used for the assay were MES, pH 6.4-7.2; Citrate-Phosphate, pH 5.1-5.9; Cacodylate, pH 6.8-7.2 ; Tris-Hcl, pH 7.9-8.6 .

(A)

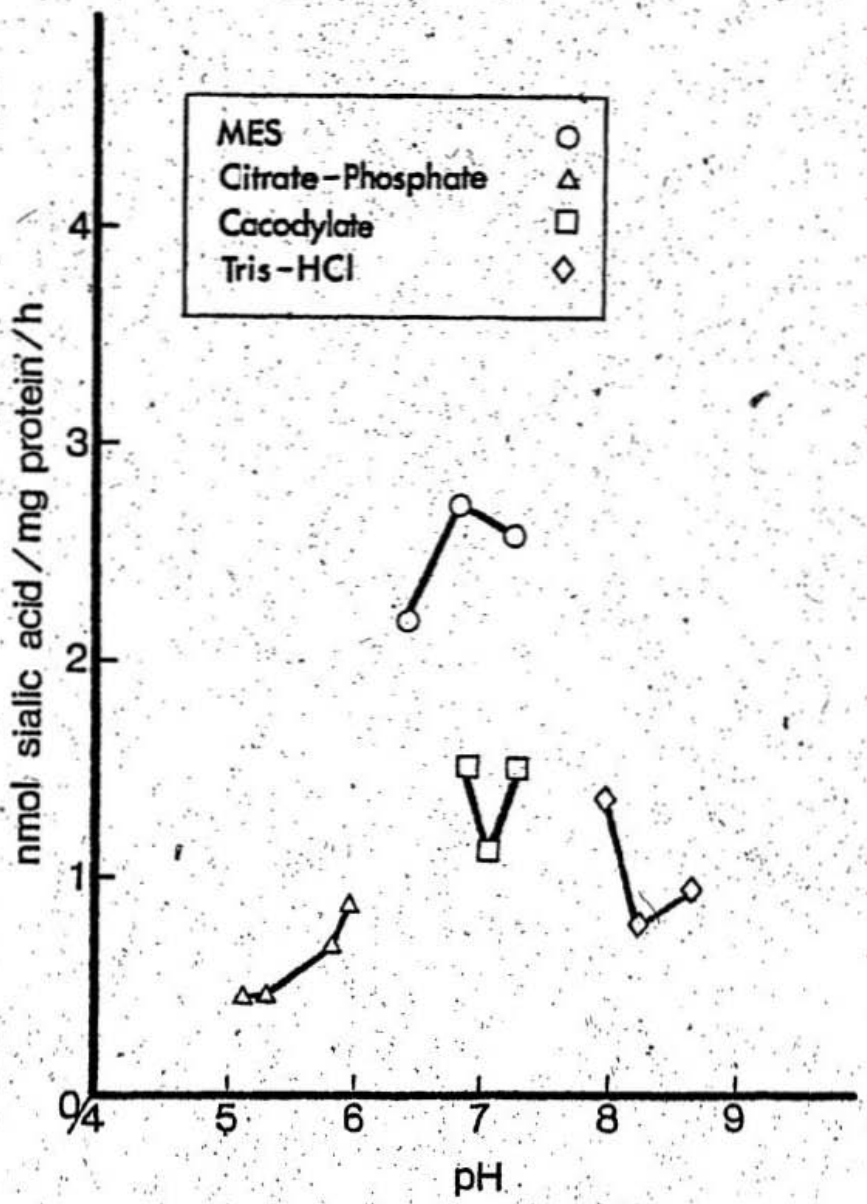


Figure 10. pH Optima of Sialyl- (A) and Galactosyl (B) transferase Activity in Lung Microsomes.

(B)

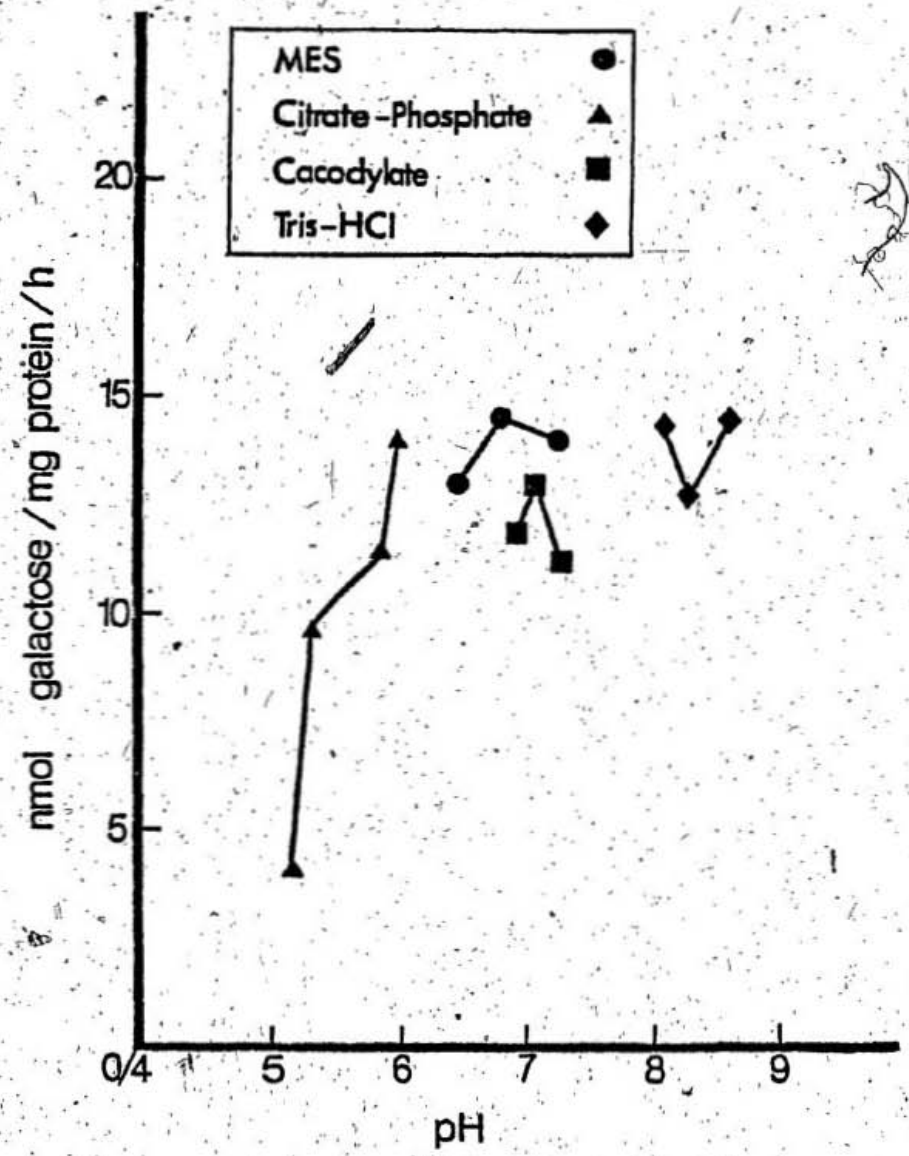


Figure 10. pH Optima of Sialyl- (A) and Galactosyl (B) transferase Activity in Lung Microsomes.

D. ACCEPTOR CONCENTRATION

In figure 11, A and B, the effects of varying the dose of different exogenously added glycoprotein-acceptors on the glycosyltransferases, are illustrated. For galactosyltransferase and especially sialyltransferase, fetuin, depleted of the appropriate sugars stimulated the highest activity. The acceptor sites of both DS—and DSG-fetuin were saturated at a high acceptor-enzyme protein ratio (3:1). Table 3, section B, shows that for galactosyltransferase a 250 µg dose of DSG-fetuin produced a 33-fold stimulation over endogenous acceptor activity alone, while sialyltransferase gave only a 6-fold increase in activity. More endogenous acceptor activity associated with the sialyltransferase, as compared to galactosyltransferase may be indicated in rat lung microsomes.

E. CATION REQUIREMENTS

Galactosyltransferase activity associated with the addition of different concentrations of Mn^{2+} is show in Figure 12, and in Table 3, section D.

Maximal stimulation occured at a concentration of 12.5 mM. Other divalent cations were tested at this dose, but they could not replace the absolute requirement shown for Mn^{2+}. The presence or absence of this cation produced no change in the specific activity of sialyltransferase as had previously been observed in ammiotic fluid (73).

(A)

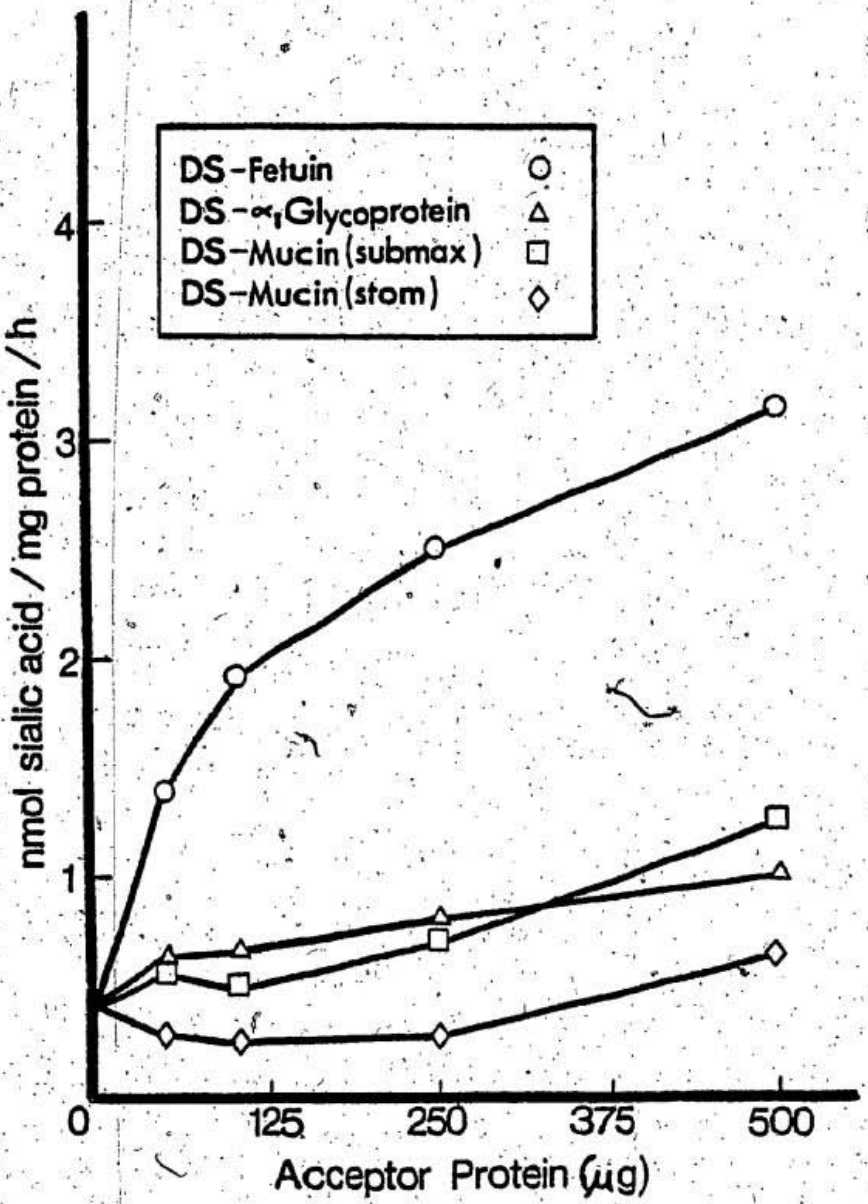


Figure 11. Sialyl- (A) and Galactosyl (B) transferase Activity in Lung Microsomes as a Function of Acceptor Protein in µg.

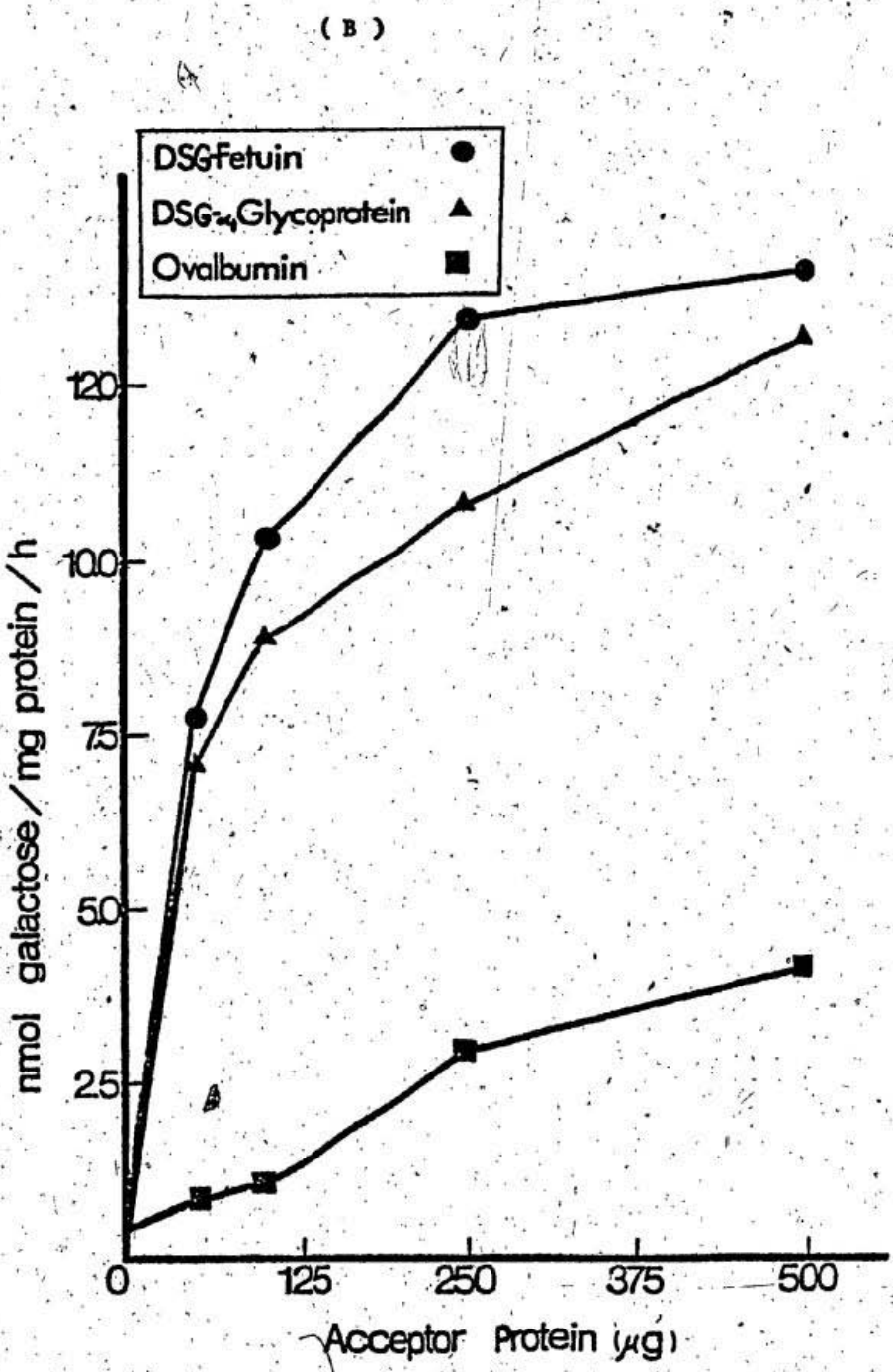


Figure 11. Sialyl- (A) and Galactosyl (B) transferase Activity in Lung Microsomes as a Function of Acceptor Protein in μg.

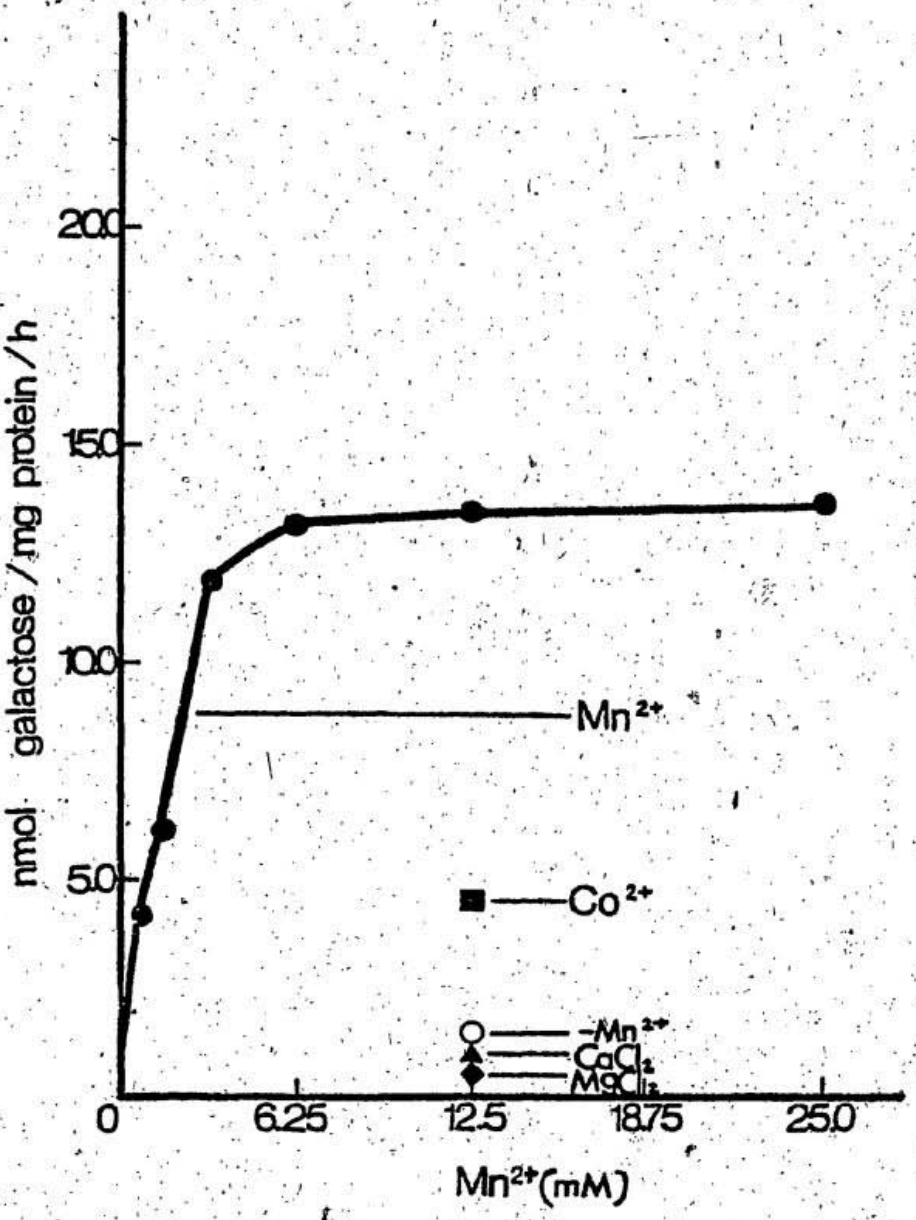


Figure 12. Cation Requirements of Galactosyltransferase Activity in Lung Microsomes.

F. EFFECT OF VARYING SUBSTRATE CONCENTRATION

Figures 13, A and B, illustrate the effect of varying the concentrations of sugar-donor substrates, CMP-sialic acid and UDP-galactose on each glycosyltransferase activity, respectively. The substrate concentration was varied in the presence and in the absence of Triton X-100. The results of the saturation curves were plotted on the basis of Michaelis-Menten kinetics according to the method of Lineweaver and Burk (50) and they are shown as inserts in these figures. The Km and Vmax values obtained are given in Table 3, section C. The remarkable stimulatory effect of Triton on galactosyltransferases is evident at all concentrations of the substrate tested (Fig. 13 B). The apparent Km value for UDP-galactose was calculated to be 0.053 mM. There was very little change in the Km value when the enzyme was assayed in the presence or absence of Triton. However, Triton had a profound effect on the Vmax of the enzyme, which was increased six-fold, from a value of 0.33 to 2.0 in the presence of 0.75% Triton X-100. Little change in either the Km or the Vmax was observed for sialyltransferase when the effect of Triton was plotted. All subsequent assays used sugar-donor substrates is near saturating amounts.

(A)

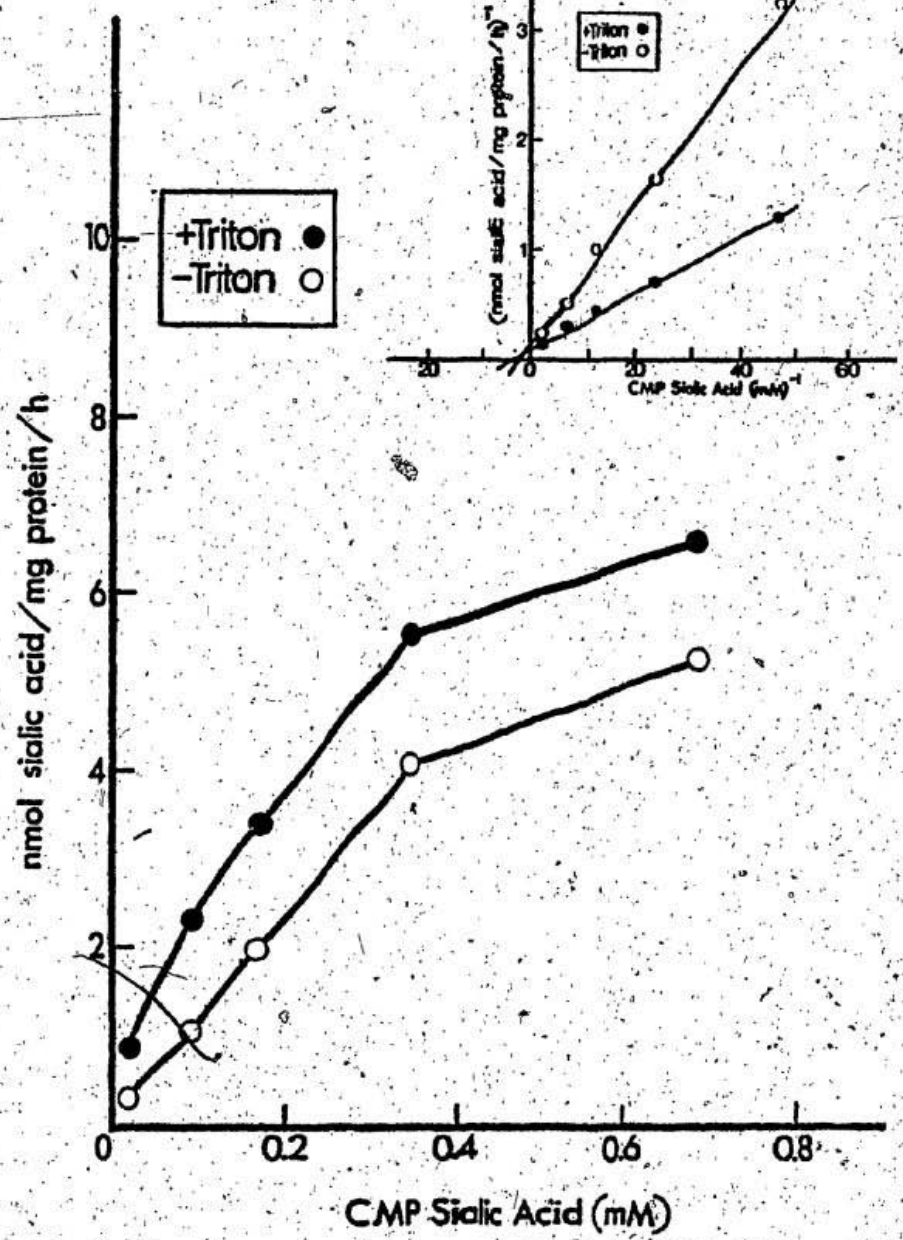


Figure 13. Sialyl- (A) and Galactosyl (B) transferase Activity in Lung Microsomes as a Function of Sugar Nucleotide Concentration.

(B)

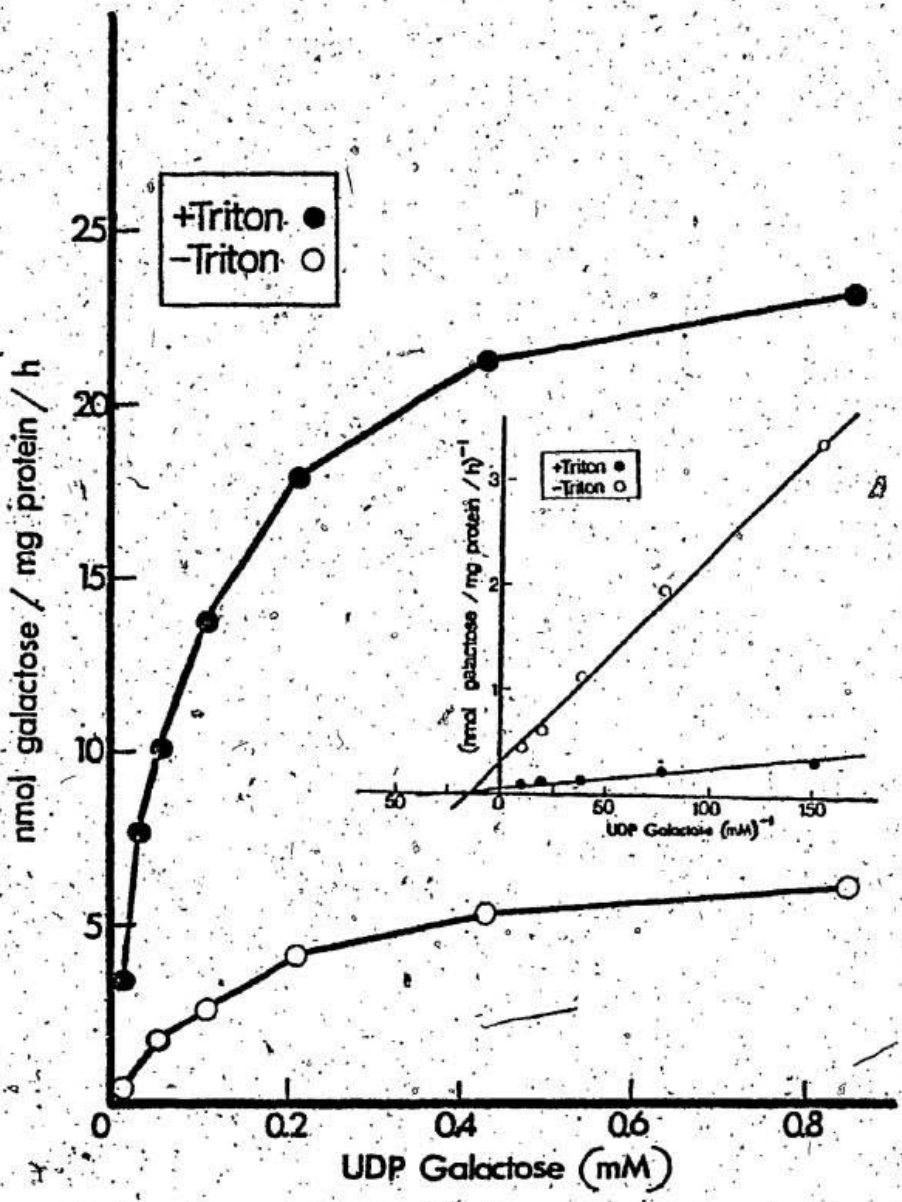


Figure 13. Sialyl- (A) and Galactosyl (B) transferase Activity in Lung Microsomes as a Function of Sugar Nucleotide Concentration.

III. EFFECT OF DETERGENTS AND PHOSPHOLIPIDS ON SIALYL- AND GALACTOSYLTRANSFERASE ACTIVITIES IN LUNG MICROSOMES.

A. TRITON STUDIES

Initial experiments using rat lung microsomes illustrated a marked difference in the glycosyltransferases' response to the detergent Triton X-100. Inclusion of Triton in the assay led to an 8-fold stimulation of galactosyltransferase, as compared to only 2-fold with sialyltransferase (Table 2). Enzyme activity plotted against varying Triton concentration is shown in Figure 14. A strong requirement of both enzymes for the detergent, ie., Triton X-100, has been determined in past studies using liver, kidney and intestine tissue (66,45,74). Figure 14 gives a different picture for lung tissue. Galactosyltransferase required a concentration of 0.75% (in final assay volume) Triton to produce the 8-fold stimulation in lung homogenate and microsomes. Sialyltransferase, on the other hand, required only 0.3% Triton to produce its maximum increase (2-fold) in activity. In addition to the small Triton requirement, sialyltransferase was virtually unresponsive to varying concentrations of Triton (Figure 14). In the same figure galactosyltransferase illustrates a more characteristic dose-dependent response. If the enzymes' contrasting behaviour toward the detergent action of Triton X-100 signifies a difference in the soluble nature and/or membrane localization, then it is also interesting that sialyltransferase activity increases only 2-fold when microsomes are isolated from total lung homogenate, whereas the galactosyltransferase increases 6-fold in its activity.

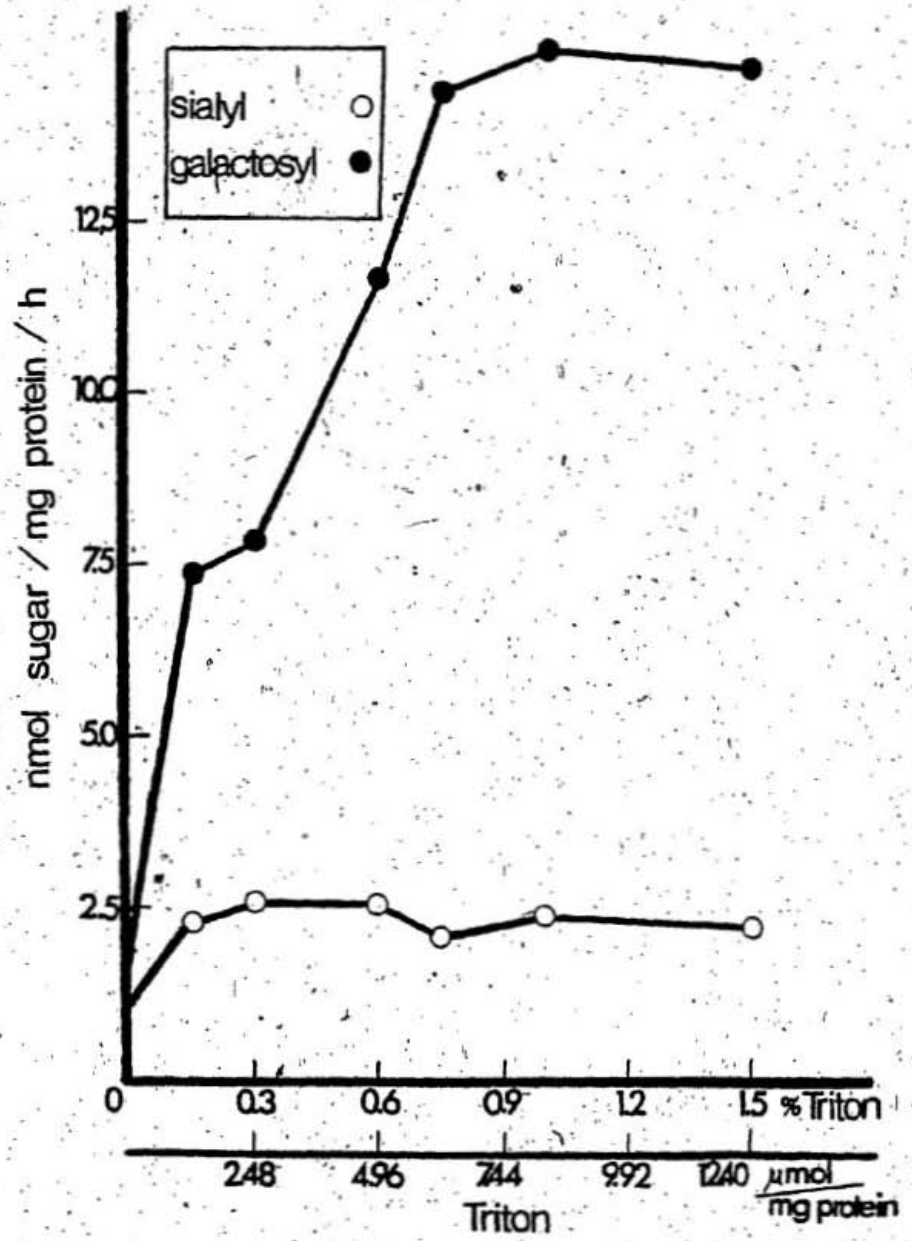


Figure 14. Glycosyltransferase Activity in Lung Microsomes as a Function of Triton X-100 Dose.

B. LIPID STUDIES

Past studies on liver (66), intestine (74) and kidney (45) have shown that lysolecithin, when added in increasing concentration greatly stimulates both galactosyl- and sialyltranferase enzymes through detergent-like action. The shape of the curve was sigmoidal for all species of lysolecithin. Since the lysolecithin effect was first claimed to result in a specific activation for the glycosyltransferase enzymes, more detailed investigations have been conducted in liver tissue (63-66). The authors concluded from these studies that the glycosyltransferase stimulation was due to the specific detergent-like effects of the entire lysolecithin molecule, with the appropriate balance of hydrophilicity and hydrophobicity. Other phospholipids and their lyso-derivatives failed to activate these enzymes. Liver microsomes were again studied in a test run using exogenously added lysolecithin. The sample protein was first adjusted to that of rat lung microsomes. Figures 15, A and B, confirmed these earlier studies by illustrating a similar sigmoidal stimulatory effect of both enzymes with increasing lysolecithin dosage (egg and linolenoyl).

When applied to lung microsomes, a different picture has emerged. Figure 16 B shows a stimulation of galactosyltransferase by all six lysolecithin species used. The increase in the activity of the enzyme was largest when linoleoyl lysolecithin was tested at different concentrations. Specific activities measured

(A-)

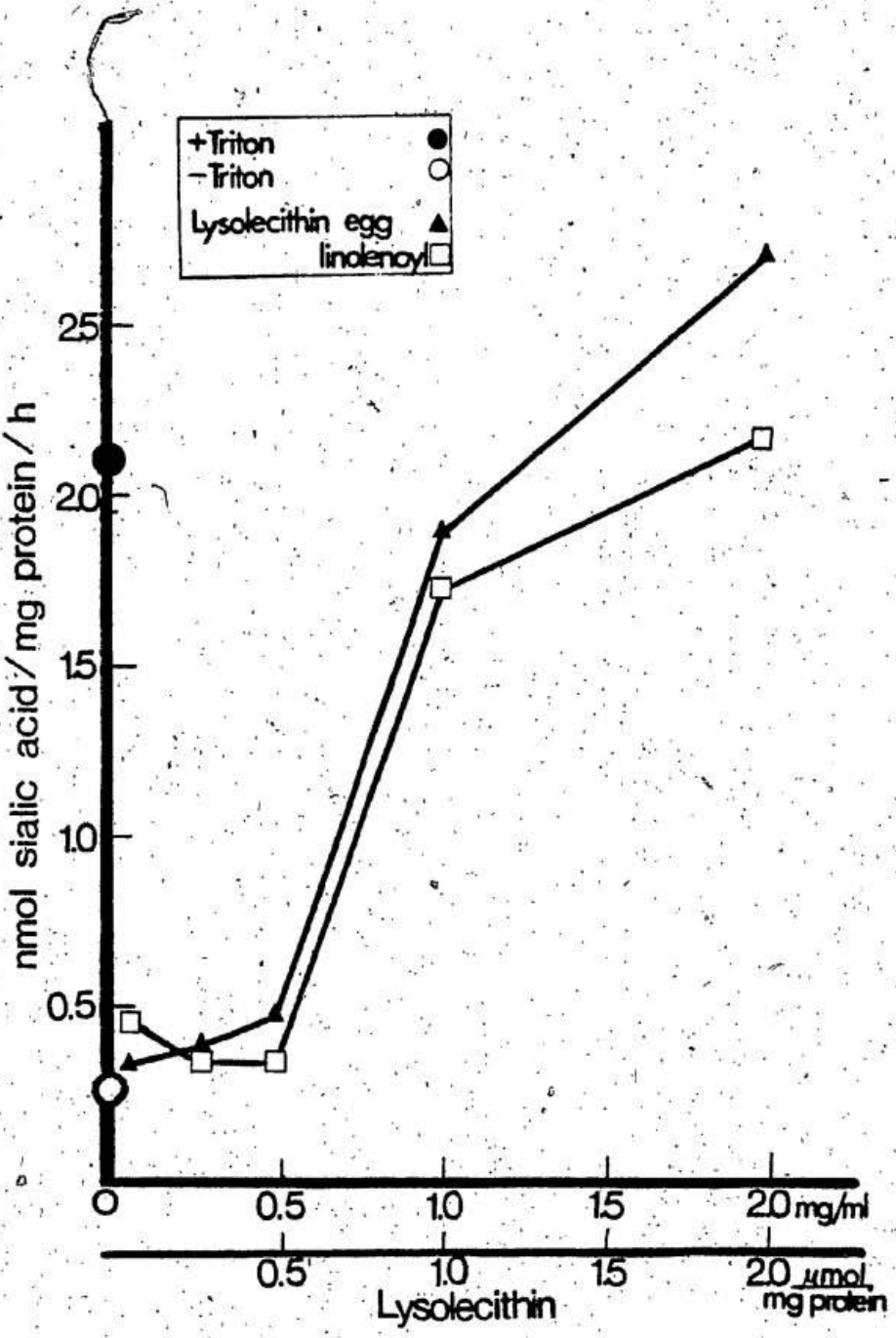


Figure 15. Effect of Lysolecithin on Sialyl-(A) and Galactosyl (B) transferase Activity in Liver Microsomes.

(B)

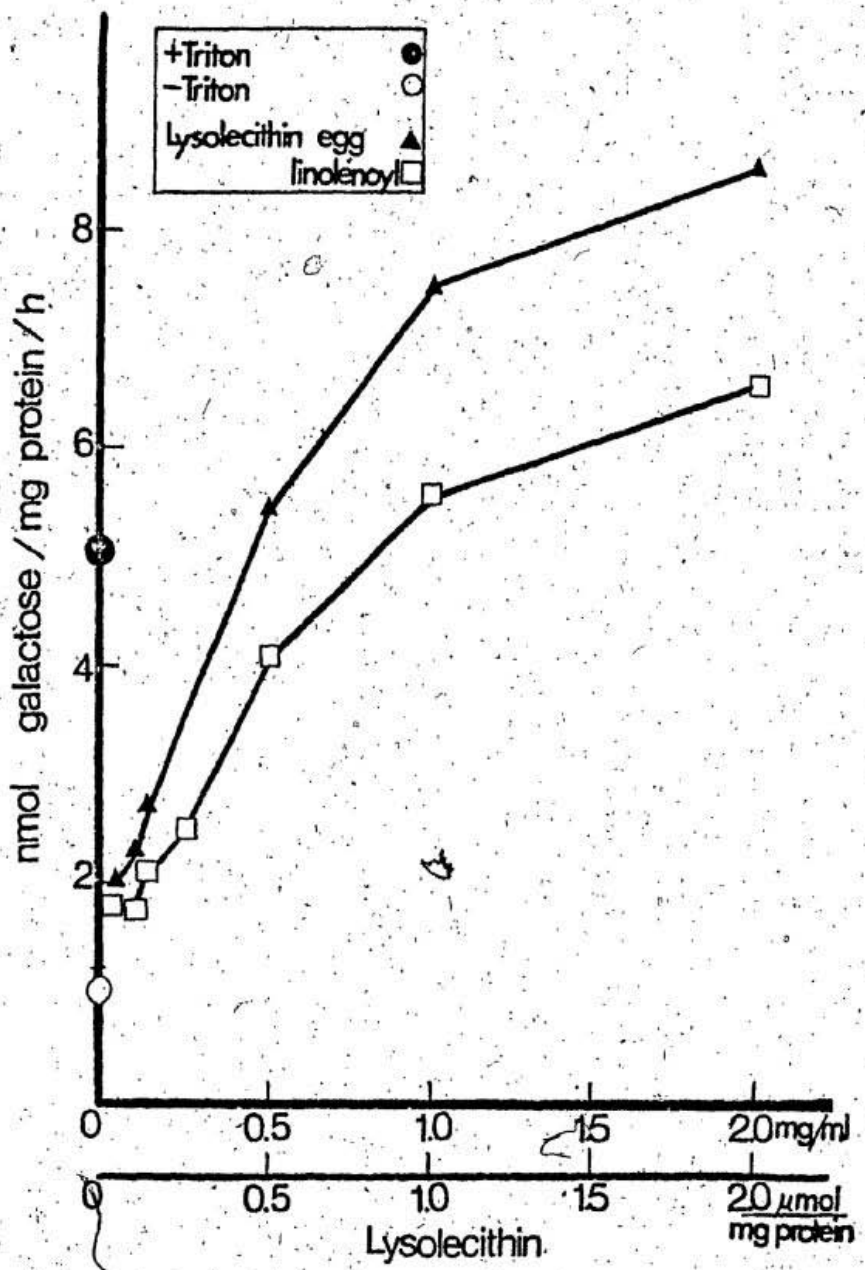


Figure 15. Effect of Lysolecithin on Sialyl- (A) and Galactosyl (B) transferase Activity in Liver Microsomes.

(A)

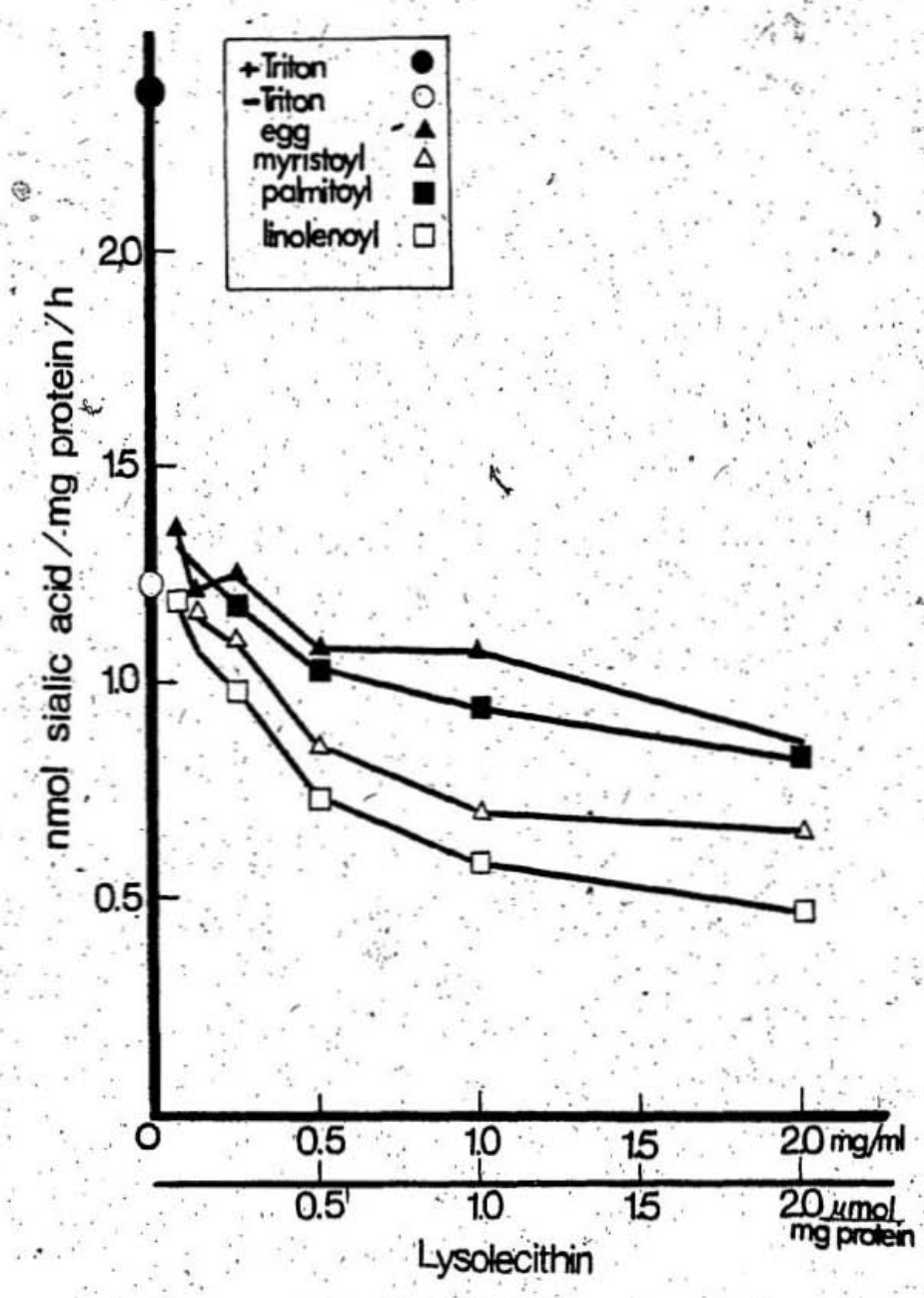


Figure 16. Effect of Lysolecithin on Sialyl- (A) and Galactosyl (B) transferase Activity in Lung Microsomes.

(B)

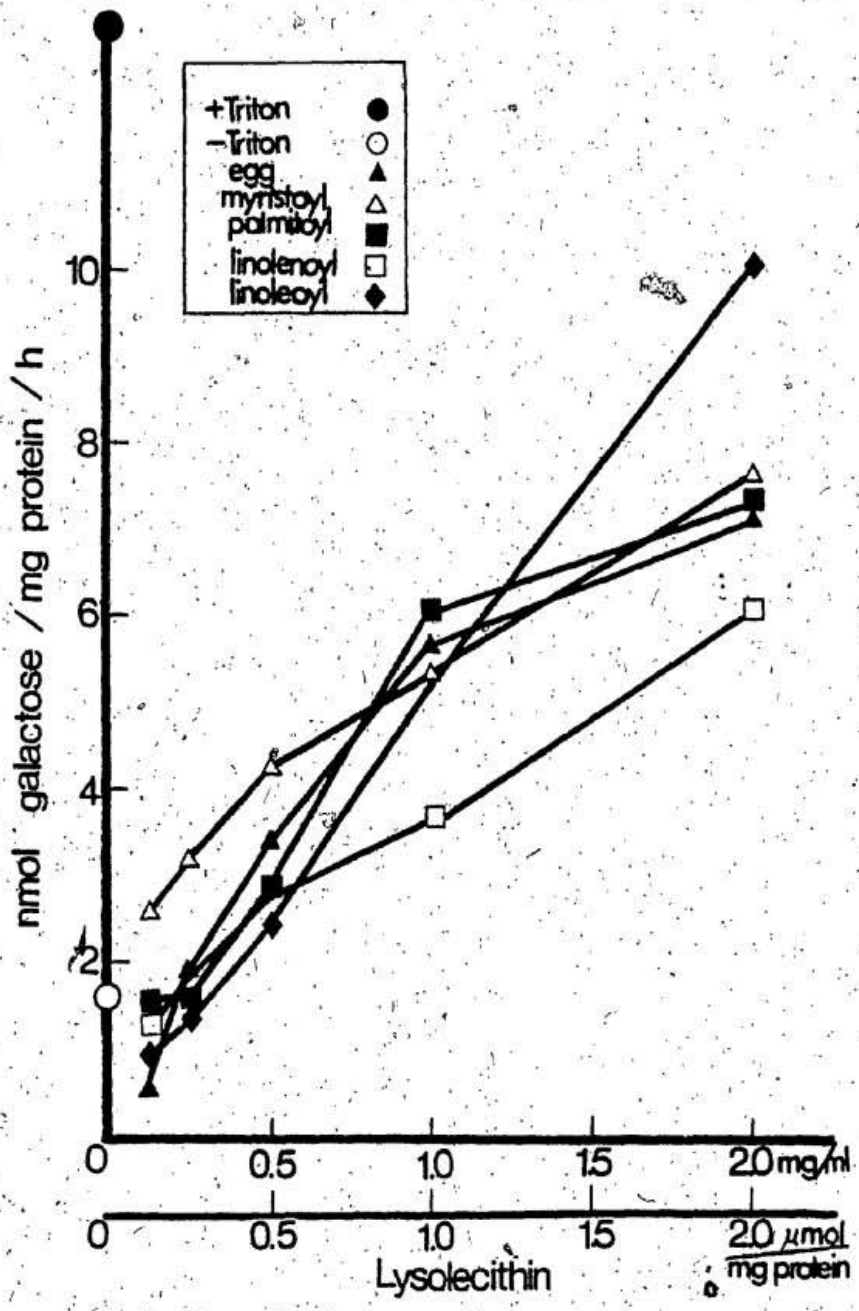


Figure 16. Effect of Lysolecithin on Sialyl- (A) and Galactosyl (B) transferase Activity in Lung Microsomes.

for this enzyme in the lung were higher than that found with liver samples, at all concentrations of lysolecithin tested. The maximal effect in lung was still less, however, than that observed with optimum Triton X-100 (0.75%). Sialyltransferase (Figure 16 A) in contrast, showed a definite inhibitory response to increasing lysolecithin doses. At the maximum 200 µg of exogenous lysolecithin a 30-40% decrease in activity was observed for all species tested (Table 4 A,B).

Table 4 (A,B) also shows the effect of various other lipid compounds on glycosyltransferase activity. All other lipid classes were either inhibitory or neutral in their effect on sialyltransferase activity, when added in 25 and 200 µg amounts. For galactosyltransferase the majority of lipids, other than lysolecithin, were inhibitory with increasing dosage. Exceptions to this were lecithin (egg, dipalmitoyl, dilinoleoyl) and PG which all showed a stimulation of the enzyme activity, although the effect was smaller than that observed with the lysolecithin family.

The role of lysolecithin, as with the mild non-ionic Triton X-100, in the activation of glycosyltransferases, is probably related to the solubilizing of the membrane (micellar-formation) and consequent enhanced interaction of the enzyme with the substrate. It is important to know if lysolecithin and Triton act the same way, on the same part of the enzyme or its membrane environment to affect activity. In some cases a

TABLE 4A

EFFECT OF LYSOLECITHIN AND OTHER LIPIDS ON GLYCOSYLTRANSFERASE ACTIVITY IN RAT LUNG MICROSOMES

Experiment		SIALYLTRANSFERASE (nmol/mg protein/h		GALACTOSYLTRANSFERASE (nmol/mg protein/h	
-Triton, - Lipid		1.25		1.65	
+Triton only +		2.43		13.5	
		Additions *		Additions	
		25 µg	200µg	25 µg	200 µg
Lysolecithin,	egg	1.24	0.84	1.84	7.12
	myristoyl	1.09	0.64	3.18	7.64
	palmitoyl	1.17	0.82	1.58	7.38
	linolenoyl	0.98	0.46	1.70	6.05
	linoleoyl	1.2	0.74	1.34	10.08
Lecithin	egg	1.19	0.99	0.83	1.37
	dipalmitoyl	1.23	1.29	0.96	2.84
	pig liver	1.22	1.18	1.24	1.05
	dilinoleoyl	0.96	0.79	2.08	3.55
Spingomyelin		1.17	0.99	1.70	1.15
PE,	dioleoyl	1.12	1.09	1.46	0.73
PS		1.05	0.92	1.42	1.33
PG		0.82	0.42	1.93	2.8
PI,	yeast	1.03	0.98	1.42	1.28
PI,	pig liver	1.18	0.81	1.83	1.60
Lyso PA	egg	1.06	0.29	2.61	1.33
	palmitoyl	0.90	0.79	1.74	1.47
	oleoyl	1.04	0.24	1.65	0.64
Palmitic Acid		1.12	0.77	1.15	0.87
Myristic Acid		0.96	0.26	0.92	0.83
Linoleic Acid		0.44	0.21	1.01	0.24
Linolenic Acid		0.34	0.11	0.96	0.73
1,2-Diolein		1.24	0.97	1.79	1.01
Triolein		1.18	1.21	1.19	0.78
Cholesterol		1.13	0.60	4.26	3.12
Gangliosides		0.92	0.54	1.33	0.55

* µg of lipid added to the assay total volume of 100 µl

+ 0.75% Triton was used in galactosyltransferase assay
0.3% Triton was used in sialyltransferase assay.

TABLE 4B

EFFECT OF LYSOLECITHIN AND OTHER LIPIDS ON GLYCOSYLTRANSFERASE ACTIVITY IN RAT LUNG MICROSOMES *

Experiment		SIALYLTRANSFERASE (Change over control)		GALACTOSYLTRANSFERASE (Change over Control)	
-Triton, -Lipid		100		100	
+Triton only		194.4		818	
		Additions		Additions	
		25 µg	200 µg	25 µg	200 µg
Lysolecithin,	egg	99.2	67.2	111.5	431.5
	myristoyl	87.2	51.2	192.7	463
	palmitoyl	93.6	65.6	95.8	447.3
	linolenoyl	78.4	36.8	103.	366.7
	linoleoyl	96.	59.2	81.2	610.9
Lecithin,	egg	95.2	79.2	50.	83.3
	dipalmitoyl	98.4	103.2	58.3	172.2
	pig liver	97.6	94.4	75.	63.9
	dilinoleoyl	76.8	63.2	126.	215.2
Spingomyelin		93.6	79.2	102.8	69.4
PE,	dioleoyl	89.6	87.2	88.9	44.4
PS		84.	73.6	86.1	80.6
PG		65.6	33.6	117.	169.7
PI,	yeast	82.4	78.4	86.1	77.7
PI,	pig liver	94.4	64.8	111.1	97.2
Lyso PA,	egg	84.8	23.2	158.3	80.6
	palmitoyl	72.	63.2	105.6	88.9
	oleoyl	83.2	19.2	83.3	38.9
Palmitic Acid		89.8	61.4	69.4	52.8
Myristic Acid		77.	20.5	55.6	50
Linoleic Acid		35.5	16.9	61.1	19.4
Linolenic Acid		27.	8.4	58.3	44.4
1.2-Diolein		99.4	77.7	108.3	61.1
Triolein		94.6	97.	72.2	47.2
Cholesterol		90.4	47.6	258.3	188.9
Gangliosides		73.5	43.4	80.6	33.3

* Control (-Triton,-Lipid) was set at 100 and all other sample (specific activities) were expressed as a per cent change in relation to this control.

delicate balance must be struck between activation by detergents and lipids and inactivation of the enzyme. In an effort to help clarify the mechanisms of action of exogenously added detergents and lipids on the glycosyltransferases, the effect of different doses of lysolecithin was studied in the presence of a saturating concentration of Triton (0.75%, 0.3%). For the galactosyltransferase enzyme, both lysolecithin and Triton individually show a strong stimulation with increasing doses. Figure 17 B illustrates that, in combination (750 µg Triton and increasing amounts of lysolecithin, up to 200 µg) the stimulatory effect of lysolecithin on the enzyme is completely masked by the Triton X-100. Figure 17 A illustrates a similar masking effect of the normal inhibitory action of lysolecithin on sialyltransferase. These masking effects would explain why any stimulatory or inhibitory action by lysolecithin was never observed in past studies, when the lipids were added to <u>in vitro</u> assays in the presence of Triton X-100.

In view of these findings lipid representatives from various classes were again added in two doses, this time in the presence of one-half the maximum Triton stimulatory dosage. As noted earlier, one-half maximum Triton dose (0.15%) produces little change in the activity of sialyltransferase. Galactosyltransferase, on the other hand, drops by almost 50% when 0.375% Triton is used instead of 0.75%. This is shown

(A)

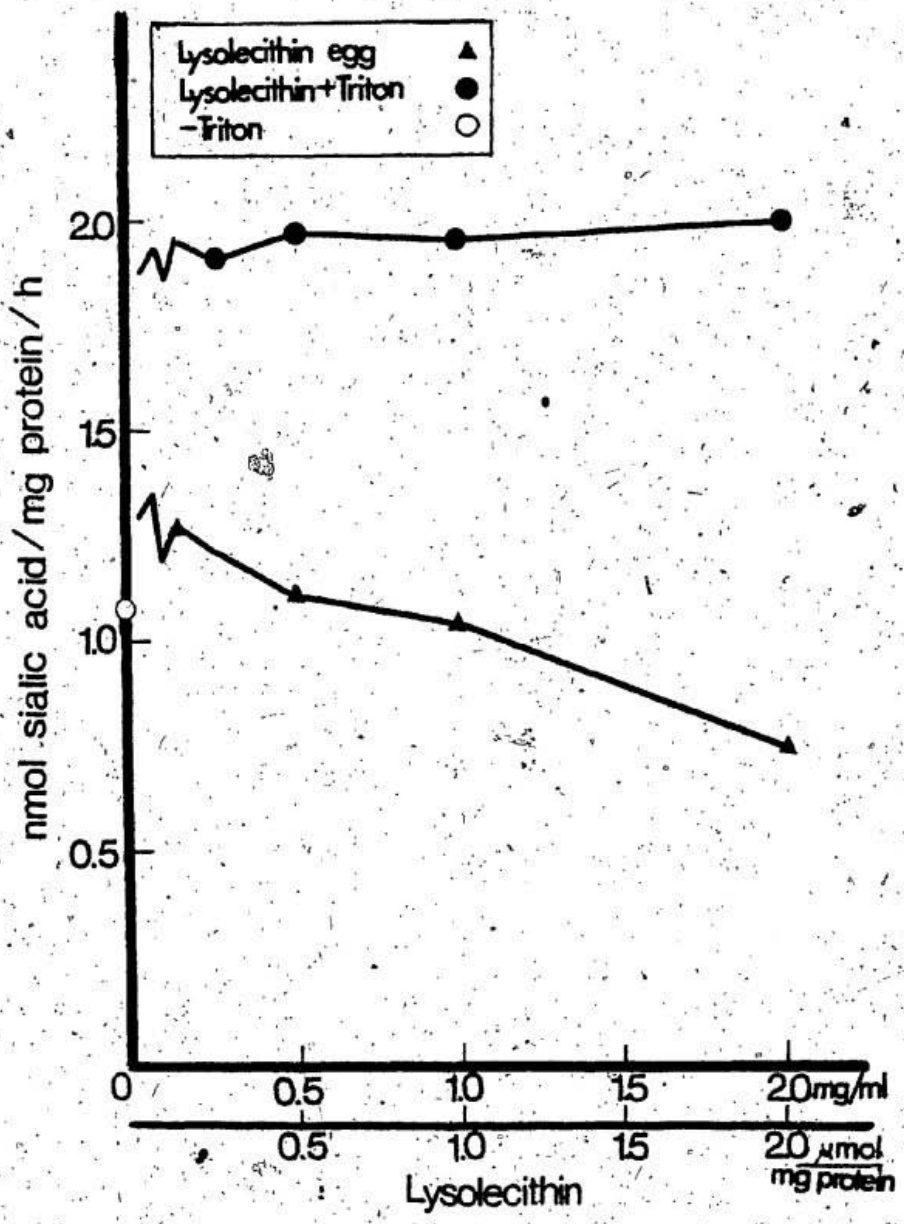


Figure 17. Effect of Lysolecithin on Sialyl- (A) and Galactosyl (B) transferase Activity in Lung Microsomes in the Presence of Triton X-100.

(B)

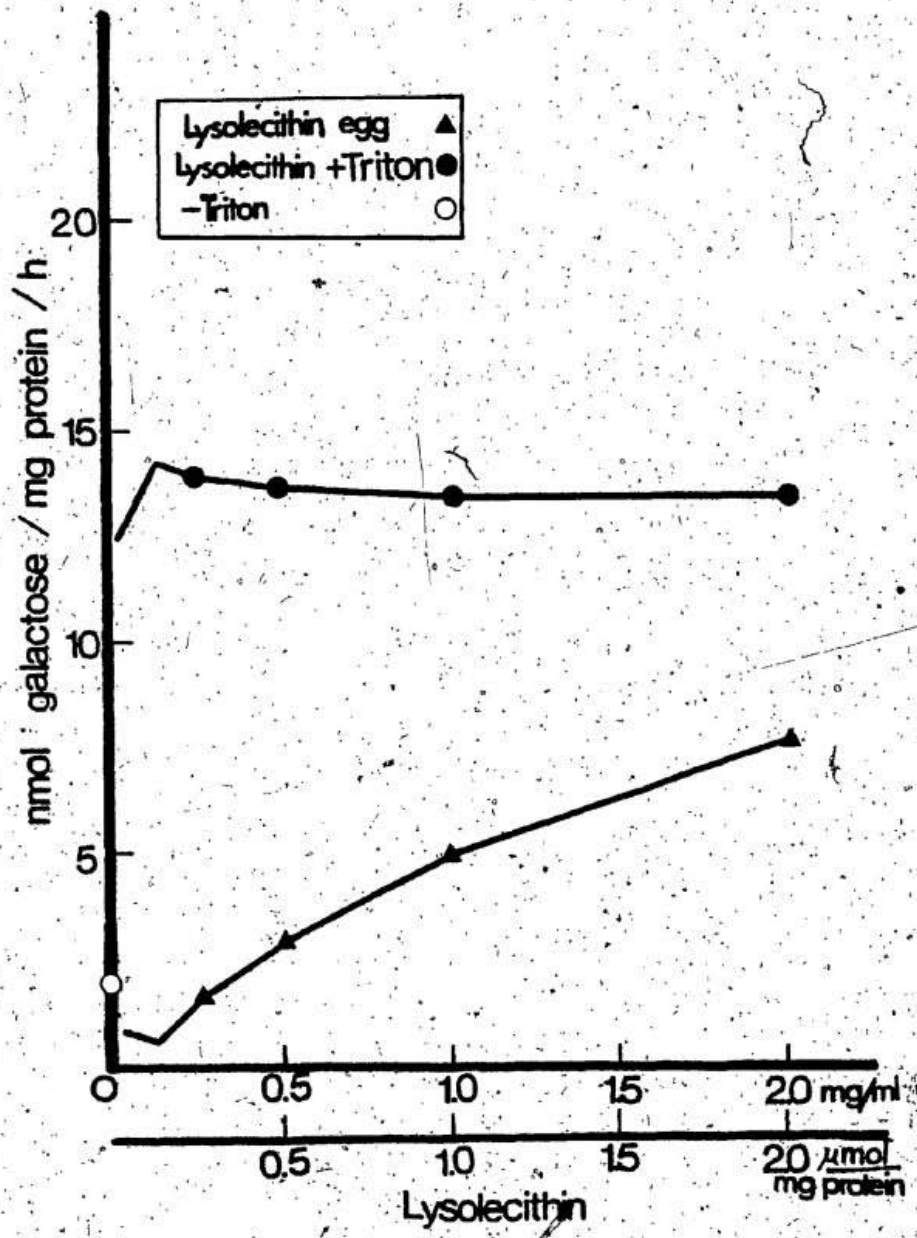


Figure 17. Effect of Lysolecithin on Sialyl- (A) and Galactosyl (B) transferase Activity in Lung Microsomes in the Presence of Triton X-100.

again in Table 5. It was previously mentioned that an additive or synergistic effect can result when Triton and lysolecithin are combined on an equal molar basis to the assay system (Pg. 22). Lysolecithin and Triton are similar in their molecular weights (536 and 635 respectively). Table 5 illustrates that when lysolecithin (100 µg, 200 µg) and a saturating amount of Triton (300 µg, 750 µg) are added together, both sialyl- and galactosyltransferase activities are again masked by the Triton. At one-half maximum Triton dose, however, a different picture emerges. For sialyltransferase, when 0.15% Triton (150 µg) is added with 100 and 200 µg of lysolecithin (linoleoyl) the resulting specific activities are nearly additive in effect, and the inhibitory response normally observed with lysolecithin alone is still seen. A one-half maximum Triton dose for galactosyltransferase (375 µg) is not similar in molar mass to the 100 and 200 µg amounts of lysolecithin (linoleoyl) and the lipids are therefore masked by Triton. This trend is also found when lipids other than lysolecithin are added in 25 and 200 µg amounts with one-half maximum Triton. The general inhibitory trend observed with these lipids on the galactosyltransferase (Table 4 A) is not found in combination with Triton and the galactosyltransferase activity that results is that for the normal value of 0.375% Triton alone.

TABLE 5

EFFECT OF TRITON AND LIPID COMBINATIONS ON GLYCOSYLTRANSFERASE ACTIVITY IN RAT LUNG MICROSOMES

Experiment	SIALYLTRANSFERASE (nmol/mg protein/h)		GALACTOSYLTRANSFERASE (nmol/mg protein/h)	
-Triton,-Lipid	1.09		1.77	
+Triton (Maximum) *	1.97		13.6	
+Triton (½ Maximum)	1.94		7.86	
	Additions		Additions	
	100 µg	200 µg	100 µg	200 µg
Lysolecithin(linoleoyl) +Max. Triton	2.03	1.95	13.1	12.3
Lysolecithin(linoleoyl) +½ Max.Triton	2.69	2.41	8.2	9.3
	Additions		Additions	
	25 µg	200 µg	25 µg	200 µg
PCegg + Triton (½max.)	2.22	3.01	6.99	7.21
Lyso PA(egg)+ Triton(½max)	2.11	2.34	7.76	5.93
Lyso PA (oleoyl)+Triton (½max)	0.80	0.34	3.34	1.41
Lyso PA (palmitoyl) + Triton(½max)	2.17	2.73	6.75	6.38
PG "	1.87	2.49	7.32	8.40
Linoleic Acid, "	1.71	2.15	7.50	4.64
Gangliosides "	2.08	2.59	7.11	6.38

* Maximum Triton is 0.3% for Sialyl-, 0.75% for Galactosyl- ½ maximum Triton is 0.15% and 0.375% respectively.

The one exception to this trend was found with Lyso PA (oleoyl). Even in the presence of Triton its inhibitory effect from 25 to 200 µg is seen, with little influence of Triton on the magnitude of the specific activity. In this regard it is interesting that Mookerjea (68) found a marked inhibition of galactosyltransferase (lysolecithin and Triton treated) by lyso-oleoyl phosphatidic acid. This suggested that lysophosphatidic acid with an unsaturated fatty acyl moiety can interfere with the interaction between lysolecithin or Triton X-100 and the enzyme protein. Lack of any inhibitory effect of the enzyme by egg yolk or palmitoyllysophatidic acid suggested to the author that proper lipid-lipid and lipid-protein interactions to inhibit the enzyme required a membrane fluid state which was better provided by an unsaturated fatty acid species, such as oleoyl.

When lipids other than lysolecithin were mixed with one-half maximum Triton (150 µg) and tested for effects on the sialyltransferase a similar trend followed. At 200 µg of lipid, the effects on activity were additive due to the similar molar ratio between Triton and lipid. At only 25 µg of lipid, however, the sialyltransferase activity that resulted was that normally observed for Triton (0.15%) alone. As in the case of galactosyltransferase, the oleoyl species of lyso PA showed exception to these trends. Oleoyl PA illustrated its normal level of inhibition with little influence on activity due to the added presence of Triton.

IV. ISOLATION OF SURFACTANT FROM RAT LUNG LAVAGE

Increasing evidence for the presence of glycoproteins closely associated with lung surfactant lipid has come to light in recent years (8-10, 42-44, 46, 75-76). Their function in this system is still unknown, although studies of the turnover rate of pulmonary surface active material have shown that these proteins are secreted from the alveolar cell in conjunction with the secretion of the surfactant into the airspaces. These glycoproteins may therefore aid in the delivery (adsorption) of phospholipids to the air/water interface from bulk suspension. Where in the cell these glycoproteins are formed, ie., where the sugars are attached, and where and when they are brought into association with surfactant lipid is not as yet known. Are the glycoproteins added to the lipids after the lamellar body stage, or do they accompany the lipid on most of its intracellular journey? In an effort to answer these questions we looked for possible glycosyltransferase activity in lung surfactant.

Surfactant was purified according to Bhattacharyya et al (8). See Materials and Methods, Figure 5. Table 6 compares the specific activities of sialyl- and galactosyltransferase in all fractions saved during the isolation procedure. In starting lung lavage sialyl- and galactosyltransferase activities were proportional within a two hour incubation time and up to 6 µg of lavage protein (Figure 18, A to D), and both enzymes

TABLE 6

GLYCOSYLTRANSFERASE ACTIVITY IN RAT LUNG LAVAGE FRACTIONS*

LAVAGE FRACTION +	SIALYLTRANSFERASE (nmol/mg protein/h) Triton		GALACTOSYLTRANSFERASE (nmol/mg protein/h) Triton	
	+	-	+	-
Starting Lavage	2.80	2.39	4.9	0.96
750g Pellet	1.31	0.95	8.61	4.53
750g Supernatant	4.23	3.14	6.18	3.69
25,000g Supernatant	9.52	7.52	11.22	6.87
25,000g Pellet	2.91	2.73	9.18	2.13
Gradient Pellet	2.37	2.09	8.91	2.99
Gradient Interface	5.98	5.71	5.04	2.01
100,000g Supernatant	7.38	6.87	6.21	4.14
100,000g Pellet	2.31	2.28	9.54	2.37

+ Refer to Figure 5, Materials and Methods, for isolation sequence of lavage fractions.

* These studies and preparations were also conducted on rabbit lung tissue and the results followed the same trend. Only rat results are presented because they were repeated 4 x (4 experiments, 10-15 rats each) and found to be reproducible.

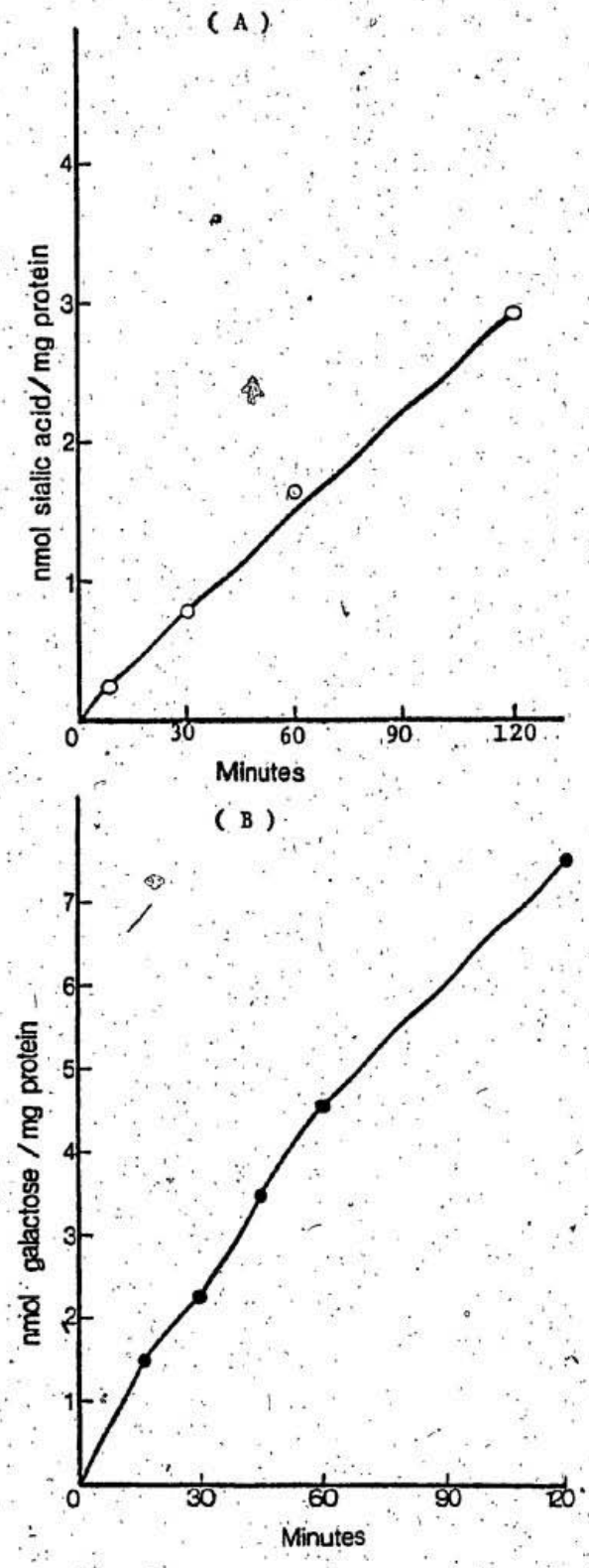
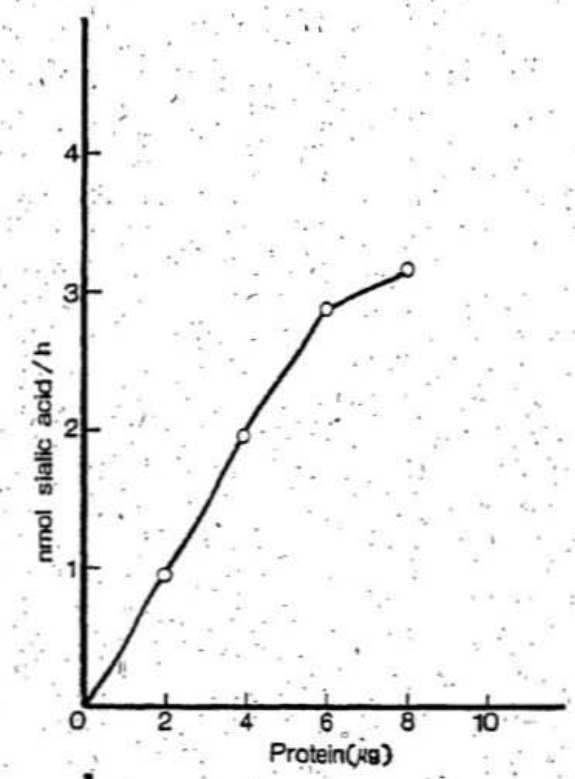


Figure 18. Glycosyltransferase Activity in Lung Lavage as a Function of Incubation Time (A,B) and Enzyme Protein (C,D).



(D)

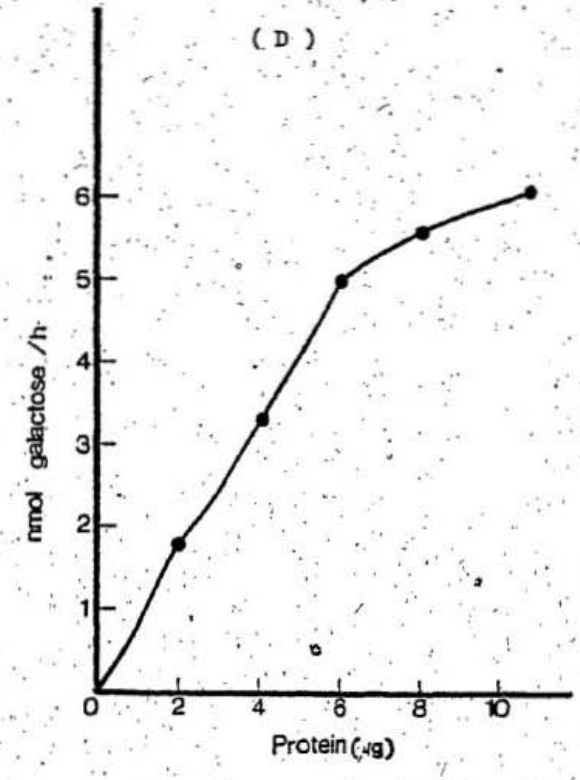


Figure 18. Glycosyltransferase Activity in Lung Lavage as a Function of Incubation Time (A,B) and Enzyme Protein (C,D).

showed good activity in this starting material. During the isolation procedure sialyltransferase activity was found highest in soluble form, whereas most of the galactosyltransferase activity was always associated with the membrane fractions. Sialyltransferase gave a consistent 3-fold increase in activity in the supernatants over the pellet fractions. Table 6 also compares enzyme activities with and without the presence of Triton X-100. Again, the same trend as in rat microsomes was observed. Addition of Triton showed little increase in the sialyltransferase in all lavage fractions. For the galactosyltransferase, however, a 4-5 fold difference in activity was observed with and without Triton, in the starting lavage, 25,000g pellet, and the final surfactant pellet. In all other fractions, a substantial increase in activity was also seen when Triton was included in the assay.

Our method of surfactant isolation was preparative rather than analytic and volumes were discarded in the various fractions. A small yield was therefore compromised for purity and 100% of each enzyme's activity may not have been recovered in all fractions. Table 6 shows, however, that both sialyl- and galactosyltransferase activities were enriched in the final purified supernatant and particulate matter respectively, in comparison to the starting lung lavage.

V. ISOLATION OF LAMELLAR BODIES FROM RAT LUNG TISSUE

Glycosyltransferase studies were extended to that of the lung lamellar bodies. Having found activity present in lung surfactant, it was of great interest to find out whether these characteristic membrane secretory structures, responsible for storage, secretion and perhaps synthesis of the various components of the surfactant system, would also have any glycosyltransferase activity. It is known that lamellar bodies are eventually secreted out of the cell into the alveolar air spaces, where they contribute surfactant lipids, glycoproteins and possibly, functional hydrolases. Increasing evidence for the presence of glycoproteins associated with surfactant lipid enhanced our interest in these lamellar bodies. These structures were isolated according to the method of Di Augustine (18), Figure 6 (Materials and Methods). All fractions were saved for further enzyme assay. Table 7 illustrates the activities for sialyl- and galactosyltransferase found in the various fractions. An enrichment of both sialyl- (11-fold) and galactosyltransferase (5-fold) can be seen in the purified lamellar bodies. In order to monitor the purity of our isolation technique, marker enzymes were assayed. Because of the very small amount of protein recovered from the pure lamellar body, final pellet assay of these subcellular fractions were limited to only a few marker enzymes in each experiment.

TABLE 7

GLYCOSYLTRANSFERASE ACTIVITIES IN SUBCELLULAR FRACTIONS OBTAINED DURING THE PURIFICATION OF RAT LUNG LAMELLAR BODIES

LUNG FRACTION*	SIALYLTRANSFERASE (nmol/mg protein/h)	GALACTOSYLTRANSFERASE (nmol/mg protein / h)
Lh	1.32	2.68
Pellet I	1.60	1.75
Supernatant I	0.84	2.23
Supernatant II	0.90	2.4
Resudial Pellet I	1.94	3.08
Pellet Wash I	0.92	2.58
Supernatant III	0.71	1.53
Residual Pellet II	1.41	2.96
Pellet Wash II	5.08	7.63
Gradient Pellet	6.7	6.25
Gradient Interface	9.41	8.83
20,000g supernatant	12.7	10.8
20,000g Pellet	14.23	13.78

* Rabbits were also used in this study, and, although not included, the results were similar to rat data presented here. Refer to Figure 6 for isolation sequence of lung fractions.

- Results shown represent the average of four separate experiments, 10-15 rats/experiment).

Table 8 summarizes the results obtained from studies of marker enzymes of selected subcellular fractions obtained during lamellar body purification. The values reported here are lower but comparable to those obtained by Di Augustine (18) using rabbit lung preparations. The specific activity of acid phosphatase was maximum in the final purified lamellar bodies (2.5-fold increase in '20,000 pellet over that of RP_2). Low concentrations of succinic-cytochrome C reductase in the final lamellar body fraction indicated that mitochondrial contamination was minimal. No measurable NADPH-cytochrome C reductase or lactate dehydrogenase could also be found in these highly purified fractions. Alkaline phosphatase, G-6-P and 5'-nucleotidase which usually give maximal localization in the lamellar body fraction were not studied.

TABLE 8

ACTIVITY OF MARKER ENZYMES IN LAMELLAR BODY SUBCELLULAR FRACTIONS *

MARKER ENZYME	SUBCELLULAR FRACTION		
	S2	RP_2	20,000 Pellet.
ACID PHOSPHATASE	5	152	386
SUCCINATE CYTOCHROME C REDUCTASE	3	10	2
NADPH-CYTOCHROME C REDUCTASE	28	4	1
LDH (Lactate Dehydrogense)	49	5	—

* Activity is expressed as nmol substrate utilized per-minute per mg of protein. Values are the average of duplicate analyses of rat lung subcellular fractions.

VI. POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis was carried out on lung lamellar body and surfactant fractions in order to extend numerous past studies (9-10, 43, 44, 46, 75) indicating the presence of specific glycoproteins in these systems. This was of particular importance in our work, where high glycosyltransferase activities were detected in both lamellar bodies and lung lavage/surfactant. Figure 19 illustrates the protein and carbohydrate staining patterns of various lung fractions subjected to gel electrophoresis. Davis gel electrophoresis (Figure 19 A) was run in order to view the overall sample protein content. Lamellar bodies, lung lavage and surfactant samples all revealed similar protein staining in the lower molecular range. Lung lavage illustrated many additional minor bands throughout the gel.

Figures 19 B,C show SDS-gel electrophoretic runs for the staining of proteins and carbohydrates, respectively. Molecular weights were plotted as a function of the relative mobilities calculated for each protein band according to the method of Weber and Osborn (100), as shown in Figure 20. The PWII (rich in lamellar bodies) and starting lung lavage showed a major protein corresponding in molecular weight to BSA(Fig. 19B). Purified lung surfactant (100,000 g pellet) and purified lamellar bodies revealed a protein of molecular weight 64,000. Both purified fractions of lamellar bodies and surfactant revealed a major protein band of molecular weight 34-36,000.

This band was also seen in the PWII and lavage fractions. In addition, these latter two fractions demonstrated intermediate minor protein bands in the 45-65,000 molecular weight range. Lamellar bodies, lung lavage and purified surfactant all gave minor protein bands between 23,000 and 28,000 molecular weights.

Diffuse carbohydrate bands were found in the 15-25,000 molecular weight range for lamellar bodies (19C) and in the 25-35,000 range for purified surfactant (19 C).

LEGEND FOR FIGURE 19

A. Davis Gel Electrophoresis

20-26 ug sample protein applied/gel. Proteins were stained with coomassie blue.

(i) BSA standard
(iv) Lung lamellar bodies (20,000 pellet)
(v) Lung lavage
(vi) Purified lung surfactant (100,000 g pellet)

B. SDS-Gel Electrophoresis

20-25 ug sample protein applied/gel. Proteins were stained with coomassie blue.

(i) BSA standard
(ii) Chymotrypsinogen standard
(iii) PWII
(iv) Purified lung lamellar bodies (20,000 pellet)
(v) Lung lavage
(vi) Purified lung surfactant

C. SDS-Gel Electrophoresis

20-25 ug sample protein applied/gel. Carbohydrates were stained with PAS method.

(i) Fetuin standard
(ii) Ovalbumin standard
(iv) Purified lung lamellar bodies
(vi) Purified lung surfactant

x - protein band corresponding to BSA

o - 64K protein band

* - 34-36K protein band

+ - 23-28K protein band

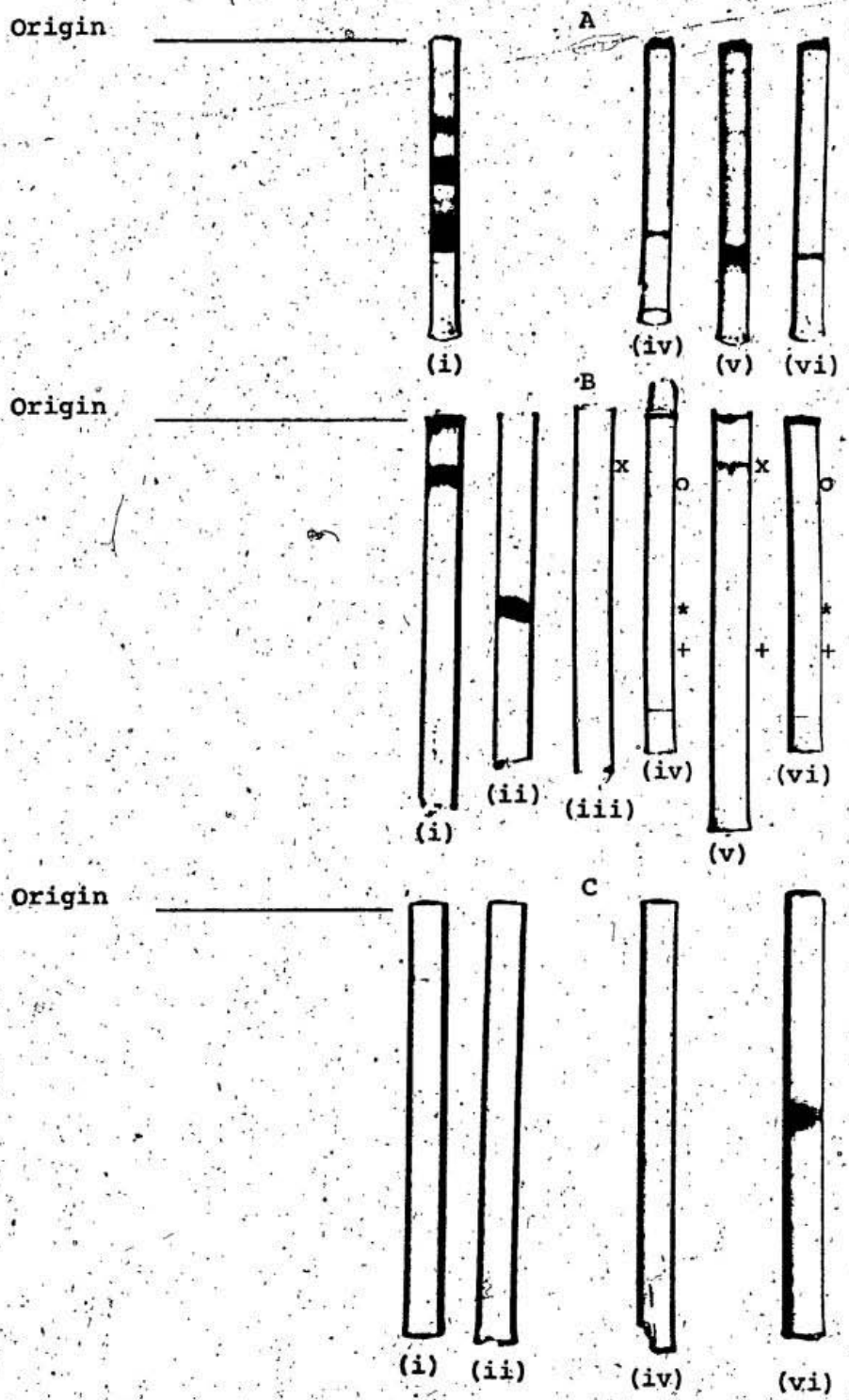


Figure 19. Protein and Carbohydrate Staining of Lung Lavage and Lamellar Body Glycoproteins.

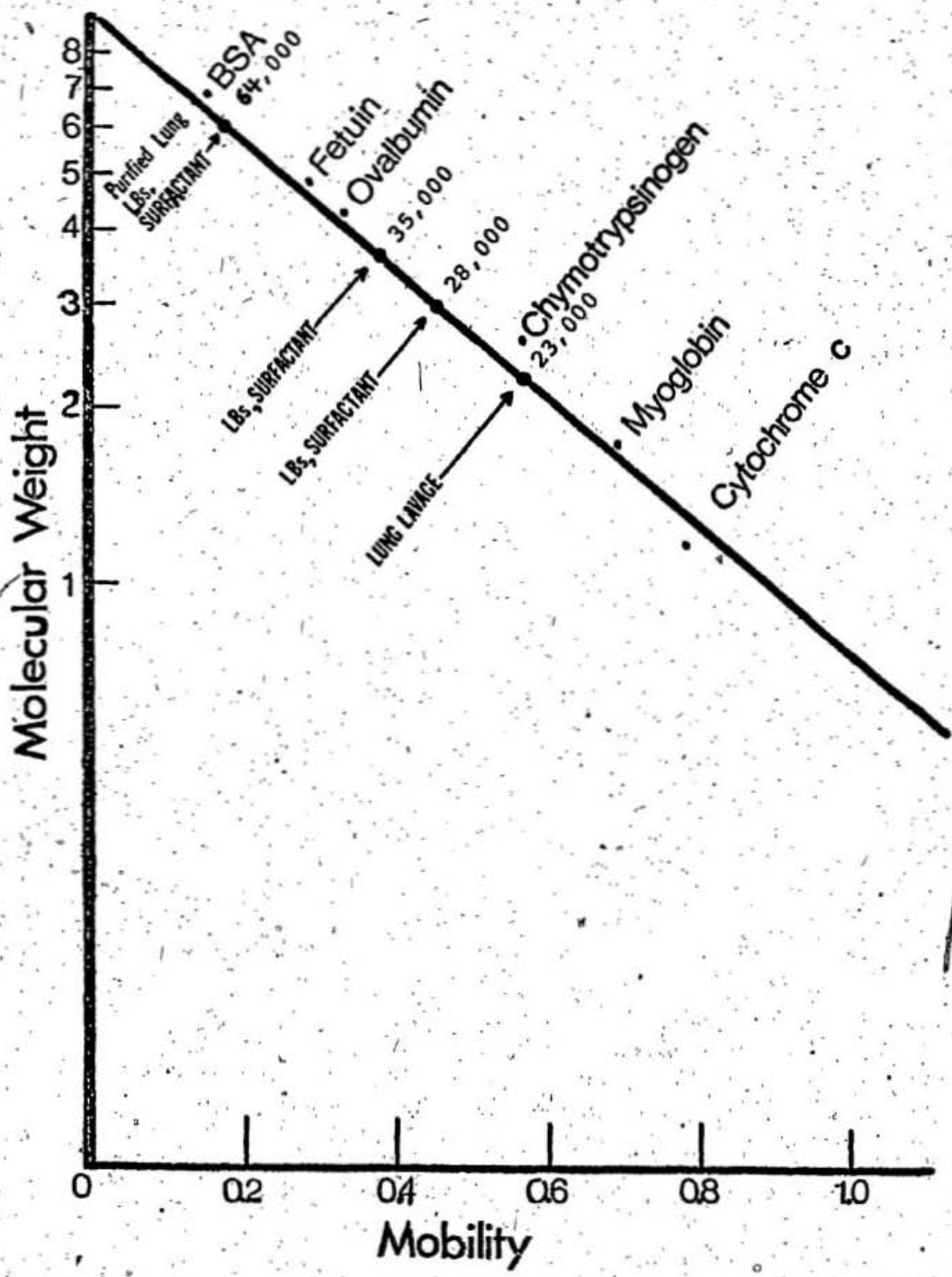


Figure 20. Molecular Weight Determination of Lung Lavage and Lamellar Body Proteins in SDS-Polyacrylamide Gels.

Although the synthesis of the carbohydrate portion of glycoproteins is not controlled by a nucleic acid template, this process is under genetic control effected through the direction of the synthesis of the glycosyltransferases by the DNA template. The glycosyltransferase activity is also controlled by substrate and cofactor availability and other environmental influences. The process of completion of carbohydrate units is regulated by the step-wise attachment of sugar residues by glycosyltransferases. As proteins pass throughout the membrane these enzymes may not always have the opportunity to act on the carbohydrate unit. The specificity of various glycosyltransferases, although high, is not absolute. The possibility of substituting one sugar for another exists, and this could contribute to heterogeneity of glycoproteins and perhaps even to serious structural alterations in diseases, in which abnormal amounts of a particular sugar nucleotide might be available. All these factors therefore stand out as logical points for rapid physiologic regulation of the synthesis and perhaps also the release of glycoproteins. They could also be loci where pathologic processes operate to control the rate of glycoprotein synthesis or to alter the nature of the product formed.

Interest of glycoproteins in medicine is increasing. There are many examples of glycoprotein alterations in certain metabolic disorders, including coronary disease, cancer and cystic fibrosis. Glycosyltransferases, however, in all these

disorders, are amenable to physiologic regulation. Because of the extremely varied functions of glycoproteins in all organisms it is important that a complete knowledge of the steps of their biosynthesis is accumulated. This should lead to elucidation of methods of control and perhaps regulation of some disease processes.

Work with cell-free systems has greatly elaborated the pathways of glycoprotein synthesis. Subcellular localization of glycosyltransferases has been carried out extensively in liver, thyroid, salivary gland, Ehrlich ascites cells, Hela cells and testis. The studies presented here are the first of its kind to examine the properties of glycosyltransferases in subcellular fractions of whole lung tissue.

Both sialyl- and galactosyltransferase enzymes in lung tissue were found to be most active with MES buffer at pH 6.8. Sialyltransferase, however, was much more specific in this requirement, while galactosyltransferase exhibited high activity over a broader range of pH and different buffers. Sialyltransferase also showed a more specific exogenous acceptor requirement than galactosyltransferase. DS-fetuin associated activity was much higher than that found using DSα_1-acid glycoprotein or DS-Mucin. DSG-fetuin was also the best acceptor for the galactosyltransferase, but only slightly above the ability of DSGα_1-acid glycoprotein. A divalent cation requirement provided by Mn^{2+} was required in the assay of galactosyltransferase as was the nucleotide ATP. The role of ATP in the assay was

especially important in the lung microsomes where UDP-galactose substrate was used in near, but not saturating amounts, and the nucleotide was therefore able to protect the sugar-donor from pyrophosphatase action.

Growing glycoproteins are located on the inside of the cell membranes. If the glycosyltransferases are also located on the inside, the sugar-nucleotide donors must permeate the membrane in order for the reaction to take place. The rate of this process will possibly change with the dynamic nature of the membrane. If, however, the enzyme bridges the membrane, or is located on the outside of the membrane the glycosyltransferase reaction itself will cause sugars to be transported across the membrane, and permeability problems will not exist. When increased permeability of sugar-nucleotide is required in the <u>in vitro</u> situation, detergents are employed in order to solubilize the membrane system.

Initial experiments using lung homogenate illustrated a marked difference in the response of sialyl- and galactosyltransferase towards the mild, non-ionic Triton X-100 detergent. Triton X-100 improves membrane solubility (substrate accessibility to the enzyme) by producing mixed micelles of lipid, protein and detergent. It binds to the hydrophobic part of the amphiphilic glycoprotein and in so doing mimics the lipid environment of the membrane interior. Only a part of the bound detergent molecules interacts directly with the protein and the rest bind cooperatively to form a micelle- like region

on the protein surface. In so doing, small amounts of Triton do not penetrate or change the conformation of the enzymes. During solubilization optimum activity occurs while the enzyme is still membrane-bound, due to an unfolding of the membrane protein chains, and in this way the orientation of hydrophobic and hydrophilic parts of the enzyme is preserved.

In lung tissue, the behaviour of sialyl- and galactosyltransferase towards Triton X-100 signifies a difference in the soluble nature and/or degree of binding of the enzymes inside the membrane. Sialyltransferase requires very little Triton (0.3%) for a maximum 2-fold stimulation of activity in lung homogenate and microsomes. Increased dosage beyond 0.3% in lung homogenate, however, led to a decline in activity. Triton X-100 produced a large stimulation of galactosyltransferase activity in lung homogenate and in microsomes (8-fold increase). A dose-dependent relationship was obtained up to 0.75% conc. which produced the highest activity. Again in lung homogenate, as with the sialyltransferase, increasing dosage beyond this maximal conc. led to a sharp decline in activity. In microsomes, higher conc. than 0.75% did not change the enzyme activity in either way.

It appears that sialyltransferase exists in much more soluble form than the galactosyltransferase. A greater percentage of activity was found in the post-microsomal supernatant than in the microsomal membranes, when these fractions were compared to the starting lung homogenate.

Galactosyltransferase, on the other hand, increased 6-fold in the microsomes over that found in lung homogenate. This, together with the fact that Triton dramatically increases the activity of this enzyme would indicate that lung galactosyltransferase is a much more deeply embedded (intrinsic) enzyme, forming tighter hydrophobic interactions in the membrane. As increasing amounts of Triton are added to the microsomes tight membrane hydrophobic interactions which act to constrain the galactosyltransferase are eliminated, and micelles are formed. In this context, it is interesting that the stimulatory effect of Triton on the galactosyltransferase was evident at all concentrations of the substrate UDP-galactose tested. Although Triton X-100 addition had little effect on the Km, the Vmax of the enzyme was increased 6-fold. Little change in either the Km or Vmax of the sialyltransferase was observed in the presence of Triton.

The decrease or levelling off of galactosyltransferase activity at Triton doses above 0.75% could be due to the total delipidation of the membrane enzyme (removal of phospholipids necessary for normal enzyme activity and conformation) or even denaturation of the enzyme. When the detergent level is increased beyond that necessary for membrane solubilization, the binding capacity of the proteins and lipids to the detergent becomes fully saturated, and mixed micelles become separated into detergent-protein and detergent-lipid. The sialyltransferase, as a much more extrinsically localized or even a near-soluble membrane enzyme, forms predominantly polar interactions

with membrane lipids or proteins, and may be released by the Triton, lipid-free. Thus, a relatively smaller amount of Triton would be required to produce the small increase in activity associated with the maximal solubility attainable. The inhibitory effect on the sialyltransferase activity in lung homogenate, at doses beyond 0.3%, may be because the Triton has a greater denaturing effect at a smaller dose with the more hydrophilic enzymes. The efficiency of Triton depends on the detergent/protein ratio and also on the amount of protein in the reaction mixture. Triton X-100, however, is not a natural detergent in the in vivo situation, and it is possible that in vitro addition alters the lipid-protein organization and therefore hampers the study of the physiological regulation of these enzymes. At very high Triton doses membrane bilayers are known to become sensitive and fragile.

Different membrane enzymes in different areas of a membrane are affected in different ways by their lipid environment. In addition, the phospholipid distribution is different in different membranes. It is important to understand this in order to learn about the mechanisms of action, physiological regulation, and how the enzymes can be affected by various agents. For an enzyme to function correctly, it requires the correct conformation of its active sites produced by the appropriate fluidity in the membrane. This is affected by different combinations of phospholipids. For example, some enzymes need only a hydrophobic environment while others require both polar and apolar parts of the lipid molecule, provided by certain head groups and fatty

acids. Localized in vivo removal and replacement of phospholipid headgroups or fatty acids could exert an allosteric control on membrane enzymes.

The effect of lysolecithin, a naturally occuring membrane lipid, with detergent-like action, was therefore tested for effects on glycosyltransferase activities in the lung. Now concentrations of lysolecithin affect most membrane-associated enzyme activities in one direction or the other. In liver tissue, the addition of lysolecithin within the physiological concentration in the cell results in a specific increase in the activities of both sialyl- and galactosyltransferase. It was concluded that the lysolecithin effect on liver microsomes was due to the surfactant properties of the entire molecule (a balance of hydrophobic and hydrophilic groups on the same molecule) and the resulting membrane solubilization.

In lung microsomes, the glycosyltransferases showed a differing response to lysolecithin. Sialyltransferase activity was inhibited and this inhibition increased with greater dosage of lysolecithin. This was true for all species of lysolecithin tested. In contrast, galactosyltransferase activity rose sharply with increasing dosage of different species of lysolecithin. Linoleoyl produced the strongest increase in activity (7-fold). This stimulatory effect was, as with liver, quite specific for lysolecithin. Most other lipid classes were neutral or inhibitory. Lecithin species (dipalmitoyl, dilinoleoyl) and PG were also stimulatory although

the effect was less than that found with lysolecithin. These observations perhaps illustrate a specific requirement for the fatty acyl and phosphorylcholine groups of lysolecithin, on the part of galactosyltransferase. Lipid species other than lysolecithin were similarly inhibitory when tested with sialyltransferase. These results illustrate a clear difference in the soluble nature and/or membrane location of sialyl- and galactosyltransferase in the lung.

In general, glycosyltransferases are very specific in their lipid requirements, whose different properties (ie. different fatty acid chain lengths and degrees of unsaturation) affect membrane fluidity. Galactosyltransferase, as a deeply embedded membrane enzyme would probably require a different phospholipid environment in vivo than that of sialyltransferase. Lysolecithin may increase the fluidity and therefore permeability of the membrane, concurrent with a less restraining effect on galactosyltransferase. In this regard it is interesting that in studies with liver microsomes (67) both PI and PS decreased galactosyltransferase activity in both lyso and Triton-affected microsomal micelles. This was due to the increased packing density of lipid fatty acid chains by PI and PS, as revealed by electron microscope.

It appears that Triton and lysolecithin work in a similar manner (same part of the enzyme or its membrane environment) to activate the galactosyltransferase. With sialyltransferase, there is a delicate balance between activation and inactivation

when detergents and lipids are employed. With regard to this situation lysolecithin is inhibitory and Triton has little effect. All lecithin species are ineffective when measured at low and high dose, whereas the more unsaturated linoleic and linolenic acids inhibit sialyltransferase and produce a very low activity. As a loosely bound enzyme sialyltransferase exists in more soluble form than galactosyltransferase. The fluidizing effect of Triton and lysolecithin may solubilize the former enzyme so that complete delipidation or membrane-dissociation occurs, with the result that enzyme activity is decreased (due to removal of lipids necessary for optimum activity).

In order to better compare the influence of the concentration of Triton and lipid, these species were mixed together. When optimal Triton dose was added to increasing amounts of lysolecithin a masking effect by Triton was seen and the activities of both enzymes were those associated with Triton alone. Lipids were then mixed with a sub-optimal dose of Triton (one-half that required for maximum stimulation) in order to decrease possible membrane disruption associated with lipid and protein separation caused by high Triton dose. This would provide a more realistic picture of lipid effects on the enzymes. When 0.375% Triton was used, lipid effects at all doses (lysolecithin and others) on galactosyltransferase were again masked by the detergent. When a sub-optimal dose of Triton (0.15%) was added to 100 or 200 µg of lipids, the affect on sialyltransferase activity was nearly additive. It is known that when lysolecithin

and suboptimal amounts of detergent (Triton) are combined, synergistic or additive effects on enzyme activity may result. One-half maximal Triton dose for galactosyltransferase 375µg, is still greater than the maximum 200 µg of lipid in combination with it. This may explain the complete masking effect observed in the case of galactosyltransferase. Another interpretation may be that, in the case of galactosyltransferase the solubilizing effect of Triton is stronger than that observed with lysolecithin. Sialyltransferase, however, is not effected to any large extent by Triton, and this more soluble enzyme may be affected more, or at least on an equal basis, by lipid addition and removal.

In this regard it is interesting that when lysophosphatidic acid (oleoyl) was combined with suboptimal Triton dosage the normal inhibitory effect on the sialyltransferase persisted, with no influence by Triton on the amount of activity obtained. The same effect was observed with galactosyltransferase, although with this tightly bound membrane enzyme, Triton had a slight influence on the inhibitory effect of lyso PA.

Thus the glycosyltransferases may be affected by phospholipids in a manner different from that of other membrane-bound enzymes. The concentration of lysolecithin, which may specifically regulate glycosyltransferase in lung, may vary in different membrane locations depending on local differences in the activity of phospholipase A_2 and acyltransferase.

The biogenesis of surface membrane is analogous to the

cellular secretory process. Multiglycosyltransferases exist in all intracellular membranes. Rapidly renewing Golgi and other membranes enriched with glycosyltransferases play a role in the rapid renewal of plasma membranes. As glycoproteins and glycosyltransferases move along the membranes, destined for export or incorporation into the plasma membrane, the membrane composition changes due to sugar addition reactions by the glycosyltransferases. Since Golgi membranes are undoubtedly the precursors of plasma membranes it is not surprising that some glycosyltransferases, may be carried from the Golgi apparatus into the plasma membrane. The biogenesis of plasma membrane is therefore linked to exocytosis (secretion) of glycoprotein into the circulation. Sialyltransferase catalyzes the transfer of the terminal sugar residue, sialic acid, to the glycoprotein acceptor chain. This enzyme, therefore, may have special significance on the recognition mechanism for membrane renewal and on the process of exocytosis.

Glycosyltransferases were studied in the lung surfactant. This system is interesting with regard to secretory processes. Lung surfactant is synthesized and packaged in the epithelial type II cell and then secreted via membranous organelles, the lamellar bodies. These structures (originating from the Golgi) may function, in addition to secretion of surfactant out of the cell, to attach the lipid, carbohydrate and protein before release.

Once out of the alveolar cell, the lamellar bodies are

rearranged into a structure called tubular myelin, whose function may be to assemble the final form of surfactant from a pool of building materials of the lamellar bodies. Surfactant is then adsorbed to the air-water interface for function. The exact chemical nature of surfactant is still not understood. The material is secreted as a lipoprotein with similar changes in both protein and lipid occuring during gestation. It is speculated that apoproteins may aid in the absorption of DPPC to the air-interface fast enough to meet physiological requirements (Fig. 2). The added glycoprotein confers less stability on the surfactant and the collapse rate of this material is more rapid (98). King (43) has isolated three proteins of molecular weights 69,000, 34,000, and 11,000. The latter two are very hydrophobic, like those found in membranes and strongly associated with lipids. Bhattarcharyya (8) has isolated three bands (36,000, 69,000, 62,000) of proteins common to both lung surfactant and lamellar bodies. The two major bands, (36,000, 62,000) contained a high percentage of sialic acid, mannose, galactose, fucose, and glucosamine, of which mannose, sialic acid, and galactose represent the greatest per cent per-peptide. These same glycoproteins have been isolated from human, dog, rabbit and chicken lungs. The greatest variation among species with regards to the 36;000 glycoprotein are the relative amount of individual sugars, especially the sialic acids (9).

The main problem in the study of this system is that

surfactant composition is found to vary with the species, age, passage through the lungs and the physiological state. The exact function of the glycoproteins associated with surfactant is, at present, an open question. Where the carbohydrate, protein and lipid are actually attached is also unknown. The nature and position of the carbohydrate may influence surfactant function and structure either intra- or extracellularly. This has therefore led to increasing interest in the specificity and turnover of transport proteins. It is suggested that the carbohydrate content facilitates the turnover of some proteins. Sialic acid and galactose seem to be interrelated in this function. Studies on the survival of human plasma α_1- antitrypsin indicate that exposure of galactose by removal of sialic acid decreases the circulatory half-life from 18 hours to 30 minutes (107).

We found high sialyl- and galactosyltransferase in purified fractions of lung surfactant. Throughout all intermediate fractions Triton was found to have the same effect on these two enzymes as were found in microsome studies. Sialyltransferase activity came out in soluble form whereas highest galactosyltransferase activity was located in the membrane fractions. In the purified lamellar bodies sialyltransferase was enriched 11-fold and galactosyltransferase, 5-fold. SDS-gel electrophoresis revealed glycoproteins of similar molecular weight as those previously isolated. A protein migrating like BSA was found in PWII (rich in lamellar

bodies) and lung lavage (containing surfactant) although this protein did not remain in the purified fractions. Only lung surfactant contained a protein of molecular weight 64,000. Both surfactant and lamellar bodies did contain a glycoprotein similar to that obtained by Bhattacharyya (8), of molecular weight 36,000.

The finding of glycosyltransferase activity in lung surfactants and lamellar bodies is very interesting. Their functions here, in relation to the secretion and functioning of lung surfactant are not known. This study represents the first finding of the activity of these glycosyltransferases in this system. Lamellar bodies have numerous hydrolases similar to those observed in lysosomes (186). Enzymes such as β-D-galactosidase, α- mannosidase glucuronidase, β-N-acetylglucosaminidase usually serve as an intracellular digestive system for the overproduction of secretory products (lipids, glycoproteins, proteins, polysaccharides). These intracellular enzymes of the lamellar bodies are secreted along with surfactant lipids into the lung lining layer. Lamellar bodies have been found to contain a higher protein/phospholipid ratio than that of alveolar surfactant. These hydrolases may therefore influence the physico-chemical state of accompanying lipid (surfactant lipid) by regulating the amount of glycoprotein components in the "glycocalyx-like" hypophase of the extracellular lining layer. The glycosyltransferases activity we have found in these lamellar bodies may function to assemble glycoproteins intracellularly, or after secretion outside the cell.

We have found a differential effect of lysolecithin and other lipids on the activation of sialyl- and galactosyl-transferase (in lung microsomes). An inverse relationship exists between the concentration of lysolecithin and lecithin in the membranes, depending on the activities of phospholipase A_2 and acyltransferase. Increasing evidence in the lung now indicates that the two palmitates contained in the DPPC of lung surfactant are not added in the initial diglyceride by de novo synthesis but more likely by the alteration of pre-existing unsaturated PC, involving phospholipase A_2 and acyltransferase (78,88,90). The lamellar bodies are found to have acyltransferase activity and they are twice as active as mitochondria or microsomes in synthesizing DPPC from unsaturated PC preformed in the microsomes (5,60).

With regard to glycosyltransferase function, the biogenesis of plasma membrane is linked to the process of exocytosis. Golgi and plasma membrane fusion to exteriorize secretory glycoproteins is well documented (14, 77). The role of phospholipase A_2 and lysolecithin in the fusion of membranes is also well known (77, 95). An increased level of glycosyltransferases in the serum of patients suffering from liver diseases has been found. The lysolecithin — lecithin cycle controlled by acyltransferases and phospholipase A_2 may prove to be an important physiological mechanism for regulating glycosyltransferases in vivo. Therefore defective lysolecithin metabolism in tissues in the disease state may be related to a hypersecretion and solubiliz-

ation of these enzymes into serum or extracellular space (increased cell turnover of cell surface).

In this context, it is interesting that Golgi and membrane fuse to exteriorize the secreted glycoproteins, and also a normal microtubule function has been found necessary for protein secretion. Colchicine, a disruptor of the microtubule system, not only inhibits exocytosis of glycoproteins from the cell, but also causes an increase in the serum level of glycosyltransferases (61b). In the lung, colchicine has been found to inhibit the active secretion of pilocarpine-induced protein secretion into a surface-active fraction of lung lavage returns (57). A decreased secretion of PC from lung slices into lung lavage was also noted after colchicine treatment (18a).

In the lung, the synthesis of the DPPC component of surfactant and the glycoprotein found associated with it may be under the physiological control of lysolecithin metabolism and synthesis. Lysolecithin may function as a membrane transducer for the co-ordinate activation and deactivation of different groups of enzymes in different parts of the cell. For example, lysolecithin affects the fluidity and permeability of the glycosyltransferase environment. Lysolecithin is also found to increase the amount of cGMP, and decrease the concentration of cAMP, in the cell (97). The exact role of lysolecithin in the co-ordinated production and secretion of the components of surfactant will be better understood when isolated type II cells are used specifically for this purpose. How and when the

glycosyltransferases found in lamellar bodies and in lung surfactant function will require further study. Their specificity of action for normal surfactant function may be an important control mechanism. Any alteration in their action may contribute to the abnormal surfactant found associated with RDS. The sialyl- and galactosyltransferases we have found in lung surfactant may be a normal constituent in this material, or, may be due to a hypersecretion of these enzymes from the plasma membrane or lamellar bodies caused by changes in lipid metabolism. It is interesting that the only lipid species to show a similar stimulation of galactosyltransferase as that of lysolecithin, were DPPC and PG. These two lipids are the main components of lung surfactant with surface-active properties. The precise relationship between the phospholipid metabolism and glycosyltransferase-dependent glycoprotein synthesis in lung tissue would require further study, although the results presented in this thesis may be considered as a beginning step towards that understanding.

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