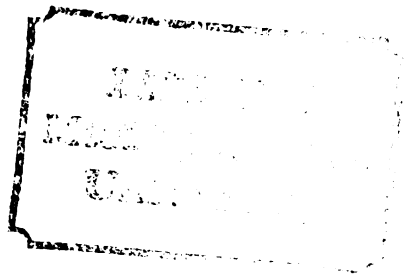

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presented by

CHRISTOPHER CHARLES MARVEL

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of the requirements for

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C C Sweeley
Major professor

Date July 2, 1980

STUDIES OF THE FUNCTIONAL ROLE AND PARTIAL CHARACTERIZATION OF A UDP-GALACTOSE:GLYCOPROTEIN GALACTOSYLTRANSFERASE

By

Christopher Charles Marvel

A DISSERTATION

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ABSTRACT

STUDIES OF THE FUNCTIONAL ROLE AND PARTIAL CHARACTERIZATION OF A UDP-GALACTOSE: GLYCOPROTEIN GALACTOSYLTRANSFERASE

By

Christopher Charles Marvel

A UDP-galactose: glycoprotein galactosyltransferase, utilizing desialylzed degalactosylated fetuin as an exogenous acceptor, was partially characterized in the rat pancreas. The enzyme activity was dependent upon manganese and detergent (Triton X-100). No evidence for a lipid intermediate between the nucleotide sugar and the acceptor protein was detected. Galactosyltransferase activity was shown to be localized almost exclusively in the smooth microsomal fraction, with a 30 fold enrichment in specific activity over the crude homogenate. No detectable galactosyltransferase activity was found in purified zymogen granule membranes.

Galactosyltransferase activity was monitored during the embryonic and neonatal development of the rat pancreas. From day 14 of embryonic development to parturition specific activity declined from 16 to 4 nmoles/mg protein/hr. A large increase (6 fold) in galactosyltransferase activity

was observed in day 3 and 4 neonates but activity declined to adult levels by day 7. Pyrophosphatase activity rose concomitantly with galactosyltransferase activity during the neonatal period. The neonatal, adult, and embryonic galactosyltransferases could not be distinguished by polyacrylamide gel electrophoresis, isoelectric focusing, or molecular sieving chromatography.

Galactosyltransferase activity was characterized in several cultured cell lines (Nil-8, Nil-8HSV, CHO, V-79, and KB) and found to have similar if not identical properties to the rat pancreatic enzyme. When cells in culture were exposed to phorbol esters, butyrate, or retinoic acid, alterations in cellular morphology were observed. Morphological changes in these cell lines may be analogous to changes occurring during development, therefore the effects of these agents were further characterized. Phorbol esters consistently elevated galactosyltransferase activity in Nil-8HSV and KB cell lines while having little effect on the Nil-8 cell line. This elevation was independent of cell density and was not related to the stage of the cell cycle. CHO cells and a cell surface mutant cell line (CHO-M) resistant to the cytotoxicity of WGA differed markedly in their response to chemical agents. CHO-M cells exhibited elevated galactosyltransferase activity for all tumor promotors tested. CHO cells were less sensitive. In

contrast, butyric acid treatment significantly increased galactosyltransferase activity in both CHO and CHO-M cells.

Most tumor promotor induced alterations in galactosyltransferase activity correlated with endogenous membrane phosphorylation in CHO and CHO-M cells. CHO cells treated with phorbol esters tumor promoters responded with little change in either endogenous phosphorylation or galactosyltransferase activity. However, CHO-M cells exhibited a significant increase in galactosyltransferase activity and a decrease in endogenous phosphorylation. A possible relationship has thus been shown between cell surface glycoconjugates, phosphorylation and galactosyltransferase activity.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
Ala	alanine
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BHKpy	polyoma virus transformed baby hamster kidney cells
BrdUrd	5-bromodeoxyuridine
CDP	cytidine diphosphate
DNA	deoxyribonucleic acid
DSG	desialized, degalactosylated
EDTA	ethylenediamine tetraacetate
EGF	epidermal growth factor
ER	endoplasmic reticulum
Fuc	fucose
Gal	galactose
GalNac	N-acetylgalactosamine
GDP	guanosine diphosphate
GIBCO	Grand Island Biological Company
GlcNac	N-acetylglucosamine
GMP	guanosine monophosphate
HEPES	N-2 hydroxyethyl piperazine-N´-2-ethanesulfonic acid
Man	mannose
MES	2-(N-morpholino) ethane sulfonic acid

NANA	sialic acid
NeuAc	N-acetyl neuraminic acid
PAS	periodic acid-Schiff reagent
PBS	phosphate buffered saline
PDA	phorbol 12,13-diacetate
PDB	phorbol 12,13-dibutyrate
PDD	phorbol 12,13-didecanoate
PMML	phorbol 12-myristate 13-acetate
PTA	phosphotungstic acid
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
Thr	threonine
TPA	phorbol 12-tetradecanoate 13-acetate
UDP	uridine diphosphate
UDP-Glc	uridine diphosphate glucose
Ve	included volume
Vo	void volume
WGA	wheat germ agglutinin
ZG	zymogen granule

INTRODUCTION

Research on complex carbohydrates has undergone an immense resurgence in the last fifteen years. Carbohydrates are no longer delegated simply to structural roles or as energy stores, but are now known to be intimately involved in multiple aspects of cellular existence. Complex carbohydrates have been implicated in such phenomena as tumorigenicity, growth control, intercellular adhesion, and morphology. The rapid progress in this field has been due to the introduction of new and improved materials and techniques that were unavailable to our predecessors. An outstanding current research area in complex carbohydrates is the integration of empirical biochemical data with their functional roles in the cell.

This thesis describes the partial characterization of a membrane-bound UDP-galactose: glycoprotein galactosyltransferase that is involved in the synthesis of glycoproteins. Utilizing this enzyme activity, a role for galactosyltransferase activity has been examined in systems that have shown potential for involvement of a glycoprotein galactosyltransferase in a defined phenomenon. The systems examined were:

1) Adult Rat Pancreas - The role of galactosyltransferases in the secretion process.

2) Embryonic Rat Pancreas - The role of galactosyltransferase in a developing tissue.

3) Normal and Transformed Cell Lines - Alterations of galactosyltransferase activity in cultured cells upon transformation.

4) Cell Surface Mutant Cells - Alterations of galactosyltransferase activity in cell lines that are resistant to specific lectin toxicity.

5) Cultured Cells - Alterations of galactosyltransferase activity upon exposure of cultured cells to tumor promotors and anti-promoters.

6) Cultured Cells - The relationship of galactosyltransferase activity to endogenous phosphorylation.

The literature review discusses glycoconjugate function and biosynthesis, the general concepts of each system, and the specific findings that have made each suitable as a model system in which to study galactosyltransferase activity. No attempt has been made to provide a comprehensive literature review for each individual system.

REVIEW OF THE LITERATURE

OVERVIEW OF GLYCOCONJUGATES

Glycoconjugates comprise a diverse class of biological compounds which contain as a defining feature covalently attached carbohydrate. These carbohydrate moietes may be as short as one unit or as large as 50 monosaccharides in some glycolipids.

Of the biologically occurring macromolecules the glycoproteins are the most diverse with the carbohydrate component ranging from 1 percent to over 80 percent of the total molecular weight. Glycoproteins are composed of a polypeptide backbone to which oligosaccharide chains are covalently attached to specific amino acid residues. Oligosaccharide chains on glycoproteins are classified according to their amino acid linkage as well as their inner-core structures. Classified separately from other glycoproteins are the proteoglycans which contain closely spaced heteropolysaccharide chains which may contain uronic acids and sulfated amino sugars. The linkage sugar in proteoglycans is commonly D-xylose attached to a seryl residue. The proteoglycans include the chondroitin sulfates, heparin, and the dermatan sulfates.

Glycolipids are generally classified into two major divisions: the glycosphingolipids and the glycoglycerolipids. The glycosphingolipids are composed of a

sphingosine base, fatty acids, and carbohydrate while the glycoglycerolipids contain glycerol, fatty acids and carbohydrate. In many instances the inner oligosaccharide cores of glycolipids are related and may be classified into families according to their tetrasaccharide core structures (1).

Glycoconjugates have been implicated as being involved in a variety of cellular phenomena including: intercellular adhesion, transformation, morphology, contact inhibition, regulation of cell growth, antigenic determination, as receptors for virus and bacterial toxins, tumorigenicity, blood group substances, etc. Present thinking delegates these specific phenomena as being mediated more through glycoproteins and glycolipids than the mucopolysaccharides, lipopolysaccharides, and proteoglycans which probably play less specific and more general roles.

Subsequent discussions will emphasize the roles and synthesis of glycoproteins over other glycoconjugates.

ROLES OF THE OLIGOSACCHARIDE MOIETY

The exact role of the carbohydrate moiety is still unclear in most instances. The complexity of the glycosylation process (and the considerable energy cost to the cell) argue for highly specific and important roles for the carbohydrate moiety. A simple general answer for their role is probably not possible. An added complexity is that

segregation of the oligosaccharide's role from other molecular structures is not always possible. For example, specificity of M or N blood group types appears to require both the presence of sialic acid as well as an involvement of amino acid residues to distinguish the M type glycoprotein from the N type glycoprotein (2). Some current ideas on the roles of oligosaccharide moieties are presented below.

Protection from Proteolytic Degradation

Glycosylation has been shown to decrease the sensitivity of certain proteins to proteolytic degradation notably adrenocorticotropic hormone (ACTH), beta-lipoprotein (3) and fibronectin (4). External beta-turns of polypeptides are quite susceptible to proteolytic degradation and a proposed general function of glycosylation may be the masking of the turn configuration (4). In one case reported, dopamine beta-hydroxylase, the removal of the terminal sialic acid residue alone resulted in increased proteolytic degradation (5).

Recognition Signal

In some cases the oligosaccharide of a glycoprotein functions as a signal for the recognition and uptake of the glycoprotein by cells of the reticuloendothelial system. A generalized role for the terminal sialic acid residues in circulating glycoproteins appears to be as a masking agent

for penultimate galactose residues. The work of Ashwell and his collaborators (reviewed in ref. 6) have demonstrated that terminal galactose residues, exposed by removal of sialic acid, constitute a configuration recognizable by hepatic cells which bind and catabolize such glycoproteins. Most long lived circulating mammalian plasma glycoproteins contain the complex type of oligosaccharide moiety ending in sialic acid. An analogous binding system has been found to operate in avian species. Many avian circulating glycoproteins have a terminal galactose residue, and its removal, which exposes the penultimate N-acetylglucosamine, results in their rapid clearance from the circulatory system.

The uptake of lysosomal glycoprotein glycosidases by cultured fibroblasts can be abolished by periodate oxidation of the glycosidases. This uptake can also be blocked by exogenous addition of a variety of carbohydrates, one of the most potent being mannose 6-phosphate. Other work has implicated a phosphorylated mannose residue of the oligosaccharide chain as the recognition signal for uptake of a variety of lysosomal enzymes (7).

Binding Sites for Viruses

Virus infection is initiated by the attachment of the virus to cell-surface receptors. Sendai and other myxo- and paramyxoviruses absorb to erythrocytes but their receptor activity is destroyed by sialidase (8). Holmgren et al. (9) have recently determined that specific binding of the Sendai

virus occurs to gangliosides containing the terminal structure: NeuAc alpha 2-8 NeuAc alpha 2-3 Gal beta 1-3 GalNAc. It is quite conceivable that similar structures in glycoproteins also function as receptors.

Blood Group Substances

The blood group substances are immunochemical specific surface oligosaccharide antigens. The defining oligosaccharides of the major blood groups are:

Group A, GalNAc alpha 1-3[Fuc alpha 1-2]-Gal beta 1-3 or 4 GlcNac;

Group B, Gal alpha 1-3-[Fuc alpha 1-2]-Gal beta 1-3 or 4 GlaNac;

Group H, Fuc alpha 1-2 Gal beta 1-3 or 4 GlcNac.

These oligosaccharides are found in saliva, gastric juice, etc. as well as on erythrocytes and may be present on glycosphingolipids or glycoproteins.

Intracellular Transport

Glycophorin A, the major integral sialoglycoprotein of erythrocytes, is incorporated normally into cell membranes when synthesis of its N-glycosidic oligosaccharide is inhibited by tunicamycin (10). In addition to its one N-glycosidic oligosaccharide it contains 15-O-glycosidic oligosaccharides whose synthesis is not inhibited by tunicamycin. Fibronectin, a major cell surface associated

glycoprotein of fibroblasts, is normally transported to the cell surface in a nonglycosylated form (11). In contrast to glycophorin A fibronectin contains only aspargine linked oligosaccharides. Studies with a viral system, vesicular stomatitis virus, have shown that transfer of membrane glycoprotein from the rough to the smooth endoplasmic reticulum is blocked by tunicamycin (12). Any analogies to a mammalian system are not known at this time.

Antifreeze Glycoproteins

Specific glycoproteins in the blood of several antarctic fish function as antifreeze agents (13). Both the carbohydrate and peptide chain are necessary for antifreeze activity, the structure being a repeated tripeptide, Ala-Ala-Thr, with a disaccharide beta-galacto-pyanosyl-(1-3)-N-acetyl-D-galactosamine attached to each threonine (14). The mechanism for lowering the freezing temperature may involve an interaction by the glycoprotein at the ice-water interface (15).

Miscellaneous Roles

Release of enveloped viruses is severely depressed when viral membrane protein glycosylation is inhibited by tunicamycin (16). Carbohydrates of immunoglobulin molecules have been shown to be required for complement-induced cytotoxicity (17).

The involvement of carbohydrate moieties in enzymatic activity is varied. The enzymatic removal of sialic acid from chorionic gonadotropin (18), follicle-stimulating hormone (19), and erythroprotein (20) leads to a loss of activity. However, incubation of pig kidney aminopeptidase with glycolytic enzymes led to no differences in catalytic activity or substrate specificity (21).

Many physio-chemical properties of glycoproteins can be related to their carbohydrate moieties. The high degree of viscosity of mucous secretions can be attributed to the acylneuraminic acids of glycoproteins (22). Similarly, solubility of many glycoconjugates is largely attributable to their carbohydrate moieties. In one instance reported, removal of carbohydrate from glucamylase I (23) resulted in reduced cold stability. The oligosaccharide may in some instances affect the three-dimensional structure of the glycoprotein, placing it into a preferred configuration.

BIOSYNTHESIS OF GLYCOCONJUGATES

Oligosaccharide chains are not primary gene products and the biosynthetic pathways for glycoconjugates proceed through a complex series of step-wise additions of carbohydrate units regulated by the specificity of enzymes acting in concert. Although the multiplicity of oligosaccharides contained in glycoconjugates is large, many share common structual features and similar biosynthetic pathways.

In nearly all instances the sugars are transferred from the appropriate nucleotide derivative. These "activated" sugars are present as: uridine diphosphate glucose (UDP-Glu), uridine diphosphate galactose (UDP-Gal), uridine diphosphate N-acetylglucosamine (UDP-GlcNac), uridine diphosphate N-acetylgalactosamine (UDP-GalNac), guanosine diphosphate fucose (GDP-Fuc), and cytidine monophosphate N-acetylneuraminic acid (CMP-NANA).

The mechanism of regulation of the biosynthesis of glycoproteins is not clear. In some systems steroid hormones appear to exert a regulatory effect on the patterns of glycoproteins synthesized by tissue culture cells (24). In one example, following partial hepatectomy, injection of hydrocortisone led to a 3-fold elevation in liver sialyltransferase (25). No elevation was found in the corresponding enzyme in the serum. Other aspects that would contribute to regulation would be availability of nucleotide sugars and compartmentalization of the glycosyltransferases.

In constrast to the precise enzymatic control that forms the classic glycoconjugates, certain proteins may undergo nonenzymatic glycosylation. Hemoglobin may be glycosylated under physiologic conditions at specific protein sites (26). This has received interest, as a two- to three-fold increase in this glycosylated hemoglobin occurs in patients with diabetes mellitus (27). Other oligopeptides can be similarly nonenzymatically glycosylated (28).

Glycolipid Biosynthesis

The major glycolipids are derivatives of the base sphingosine. Their biosynthesis begins at the C-1 hydroxy group of ceramide with the transfer of glucose or galactose from UDP-Glc or UDP-Gal. The carbohydrate chain is then extended by the step-wise addition of sugars from the appropiate nucleotide sugars without involvement of carrier lipids (29). The biosynthetic enzymes are membrane-bound, and in the liver are good markers for the Golgi apparatus (30). It must be emphasized that these addition reactions are enzymatically controlled through the specificities of the transferases present. Although large numbers of carbohydrate moieties from glycolipids have been described structual similarities indicate the presence of defined carbohydrate sequences.

Glycoprotein Biosynthesis

The biosynthesis of carbohydrate moieties on proteins involves more than the sequential addition of sugars from their nucleotide derivatives. These additional biosynthetic reactions will be described in detail below. The common covalent linkages between carbohydrate and protein occurs with five amino acids. Two of them, 5-hydroxy-L-lysine and 4-hydroxy-L-proline, occur rarely. Three of the amino acids constitute the majority of the amino acid linkages with the biosynthesis of the carbohydrate chain being dependent upon

the amino acid present.

Synthesis of O-Glycosidic Linked Oligosaccharides

The O-glycosidic linkages in glycoproteins are in the vast majority of cases to the amino acids serine or threonine. This bond is commonly characterized by its lability to mild alkali resulting in cleavage through beta-elimination. The biosynthetic route of serine and threonine linked carbohydrates is probably similar to the synthesis of glycolipids in that only sequential addition of sugars from their nucleotide derivatives is thought to occur (31). These oligosaccharides have less complex structures than those found in N-glycosidic linkages. The major alkali-labile oligosaccharide found in human erythrocyte membranes is: N-acetylneuraminyl-(2-3)-beta-D-galactopyranosyl-(1-3)-[N-acetylneuraminyl-(2-6)]-D-N-acetylgalactosamine (32). Similar structures are found in erythrocytes of other species with some variance in sialylation but with the common core unit beta-D-galactopyranosyl (1-3)-D-N-acetylgalactosamine (33). In addition to erythrocytes identical oligosaccharides are found in fetuin (34) and canine submaxillary mucin (35).

Synthesis of N-glycosidic Asparagine-Linked Oligosaccharides

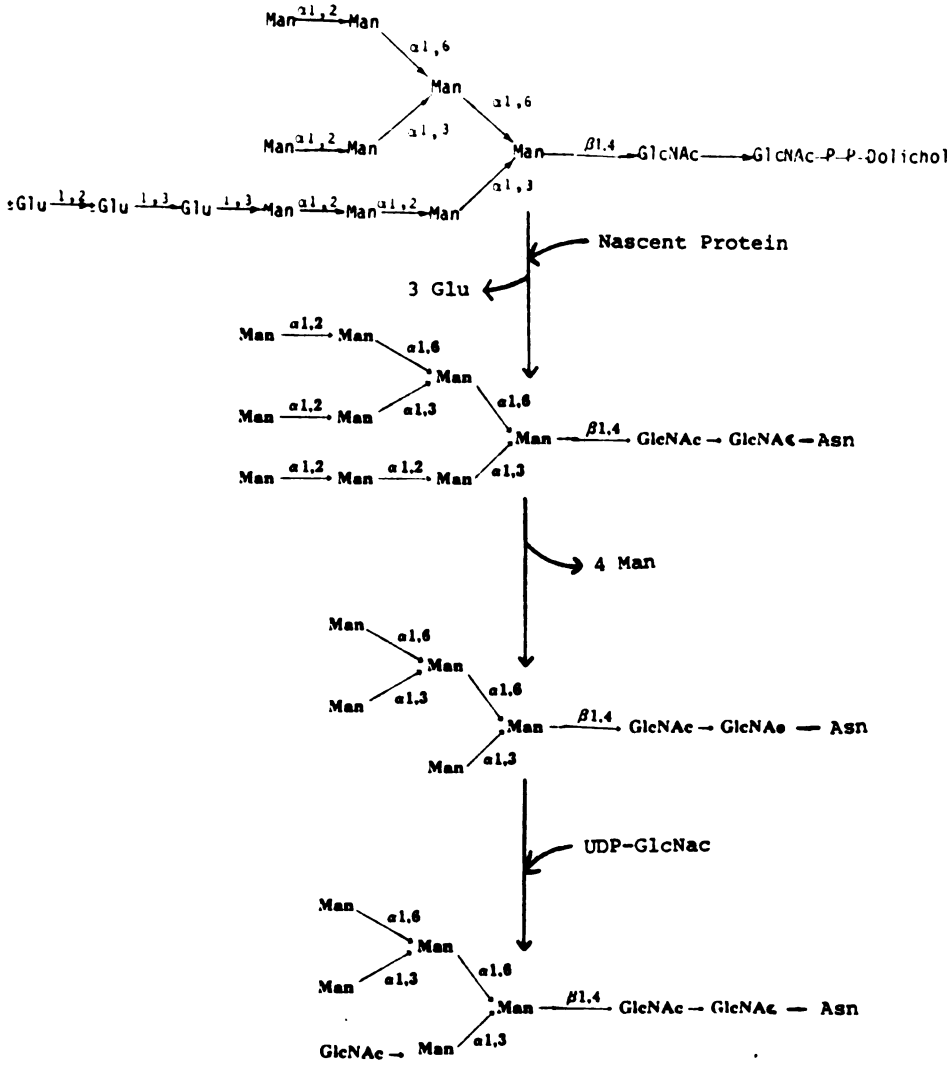
The N-glycosidically linked oligosaccharides have complex synthetic routes which are described in detail below.

Inner-Core Glycosylation - The biosynthesis of the asparagine linked oligosaccharides is initiated by the transfer of N-acetylglucosamine from UDP-GlcNac to the polyisoprenoid lipid dolichol phosphate. Sequential addition of sugars from their nucleotide derivatives to the N-acetylglucosaminylpyrophosphoryldolichol occurs to form the unit Man(beta 1-4)GlcNac(betal-4)GlcNac P-P-Dolichol. Additional mannose residues and three glucose residues are added to this structure to form the completed "inner core" dolichol linked intermediate. This complete structure is shown at the top of Figure 1. The entire oligosaccharide moiety is then transferred from the dolichol pyrophosphate to an asparagine residue on the polypeptide chain (36,37,38). This transfer is thought to occur before complete translation of the nascent chain has occurred (39). Studies of glycosylation have been greatly advanced by the discovery of a method to specifically inhibit N-glycosylation. Tunicamycin, an antibiotic from Streptolmyces lsosuperficis, specifically inhibits the N-glycosylation of asparagine residues by blocking the transfer of oligosaccharide from the dolichol intermediate to the protein core (40,41).

Processing Reactions - The glucose containing asparagine linked oligosaccharide serves as the precursor for both the polymannose and complex type oligosaccharide chains of glycoproteins (42). The asparagine-linked oligosaccharide is now processed as outlined in Figure 1,2. The

Figure 1. Biosynthesis of Asparagine-Linked Complex-Type Carbohydrates Part 1

The biosynthesis of an asparagine-linked oligosaccharide is shown. Synthesis is initiated by the transfer of a preformed oligosaccharide from a dolichol intermediate. Specific monosaccharides are then cleaved off and one N-acetylglucosamine residue is added.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α1,6
Man
α1,6
Man α1,2 Man
α1,3
Man β1,4 GlcNAc GlcNAc-P-P-Dolichol
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α1,3
Nascent Protein
3 Glu
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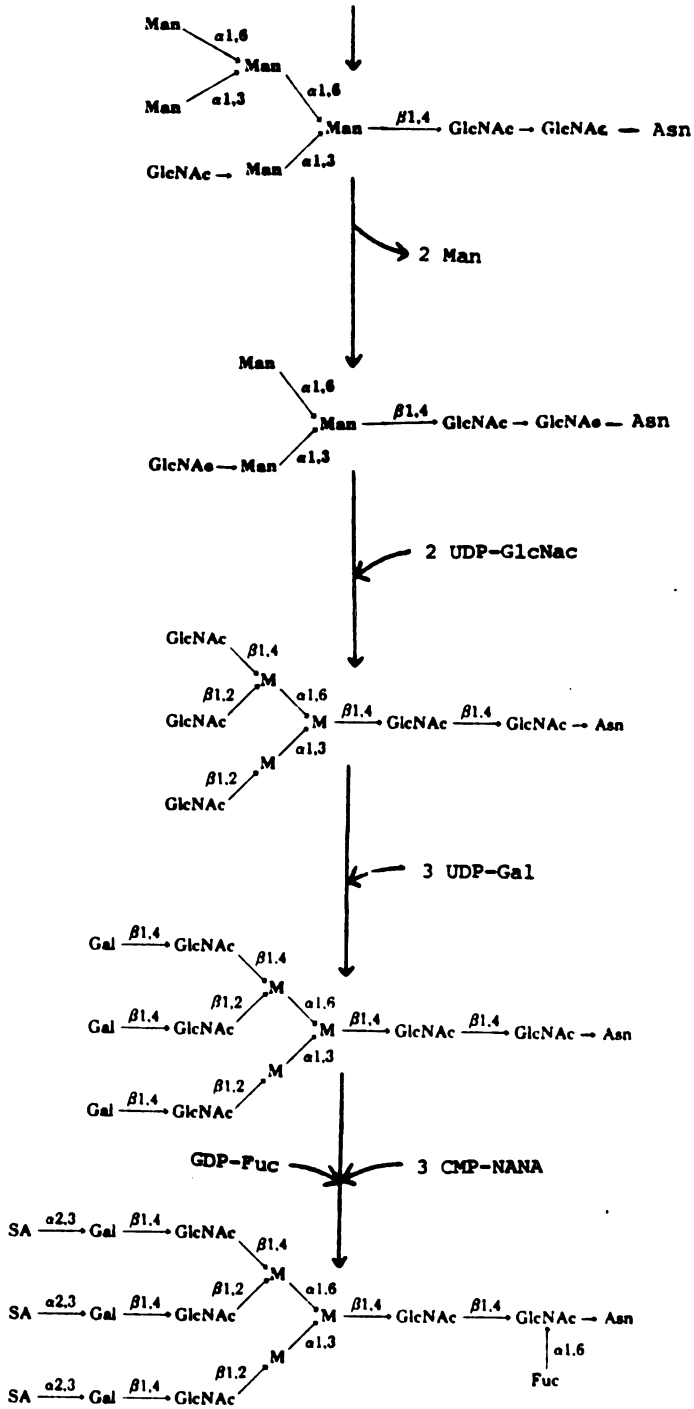
4 Man
Man
α1,6
Man
α1,6
Man
α1,3
Man β1,4 GlcNAc GlcNAc Asn
Man
α1,3
UDP-GlcNac
Man
α1,6
Man
α1,6
Man
α1,3
Man β1,4 GlcNAc GlcNAc Asn
GlcNAc Man
α1,3

Figure 2. Biosynthesis of Asparagine-Linked Complex-Type Carbohydrates Part 2

Processing of the core oligosaccharide continues with the hydrolysis of two mannose residues followed by addition of monosaccharides from their respective nucleotide sugars. The completed "complex type" oligosaccharide is at the bottom of the figure.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α1,6
Man
Man
α1,3
α1,6
Man
β1,4
GlcNAc → GlcNAc — Asn
GlcNAc →
Man
α1,3
2 Man
Man
α1,6
Man
β1,4
GlcNAc → GlcNAc — Asn
GlcNAc — Man
α1,3
2 UDP-GlcNac
GlcNAc
β1,4
M
β1,2
α1,6
GlcNAc
M
β1,4
GlcNAc
β1,4
GlcNAc → Asn
M
α1,3
β1,2
GlcNAc
3 UDP-Gal
Gal
β1,4
GlcNAc
β1,4
M
β1,2
α1,6
Gal
β1,4
GlcNAc
M
β1,4
GlcNAc
β1,4
GlcNAc → Asn
M
α1,3
β1,2
Gal
β1,4
GlcNAc
GDP-Fuc
3 CMP-NANA
SA
α2,3
Gal
β1,4
GlcNAc
β1,4
M
β1,2
α1,6
SA
α2,3
Gal
β1,4
GlcNAc
M
β1,4
GlcNAc
β1,4
GlcNAc → Asn
M
α1,3
α1,6
β1,2
Fuc
SA
α2,3
Gal
β1,4
GlcNAc

three glucose units are sequentially removed with the first excision being rapid (1 or 2 min) and the second and third occurring at decreasing rates. The exact role of the three glucose units is not known; however, several explanations have been offered. Glucose removal could serve as a control point for the rate of glycosylation of nascent proteins (43). Glucose-free and glucose-containing oligosaccharide-lipids may serve as oligosaccharide donors for different classes of oligosaccharides to nascent proteins (44). After removal of the glucose residues four mannose units are excised followed by the addition of GlcNac to an alpha-1-3 linked mannose. The addition of GlcNac is required before additional processing can occur. Subsequently two additional mannose residues are removed. At this point one or two additional GlcNac units are added to the alpha 6 linked mannose. This oligosaccharide is described as the "complex type" and may be further glycosylated in the smooth endoplasmic reticulum or the Golgi apparatus. A different type of oligosaccharide is formed if processing does not occur at the addition of the GlcNac to the alpha 3 mannose. These oligosaccharides are termed "high mannose type" and vary in structure from the simple core sequence to more extended structures formed by the addition of more mannose units.

The major envelope glycoprotein of murine leukemia virus (gp 70) contains two classes of N-asparagine linked oligosaccharides, high-mannose and complex (45). At least

in this example the viral protein structure is the primary factor in determining the mode of glycosylation.

Terminal Glycosylation - Inner core glycosylation occurs in the rough endoplasmic reticulum (RER) concurrent with or soon after emergence of the nascent polypeptide chain into the endoplasmic reticular luminal space. The polypeptide is then translocated through the endoplasmic reticulum (ER) to the Golgi apparatus where terminal glycosylation occurs (46). This glycosylation involves addition of N-acetylglucosamine, galactose, fucose, and sialic acid residues. The final glycosylation steps involve the incorporation of fucose and sialic acid residues. Current evidence suggests that the synthesis of terminal trisaccharide units (sialic acid-galactose-N-acetyglucosamine) of glycoproteins occurs by the step-wise addition of monosaccharide units from the appropiate nucleotide sugars (47). The presence or absence of specific terminal carbohydrates or variations in their modes of attachment contributes to the structural heterogeneity found in glycoproteins. These terminal glycosyltransferase activities are characteristic of the microsomal pellet and the Golgi apparatus (48). Evidence also exists for a plasma membrane localization of terminal glycosyltranferase activity (49). This localization is not without controversy (50) and the existence of cell surface glycosyltransferases is still in question.

The presence of the terminal carbohydrates of oligosaccharide chains may have active as well as passive roles. Kreisil et al. (51) has determined that the protein and carbohydrate moieties of rat membrane glycoproteins turn over at different rates. The half-life of the protein is between 70 and 80 hours while L-fucose is 12.5 hours, N-acetylneuraminic acid is 33 hours, and D-galactose is 20 hours. Consequently, not only the terminal L-fucose and N-acetylneuraminic acid, but even the penultimate galactose residues turn over faster than the protein core. This implies a late processing step of uncertain nature. After terminal glycosylation is completed the glycoprotein may be secreted or utilized by the cell as a membrane glycoprotein.

ALTERATIONS OF GLYCOCONJUGATES IN TRANSFORMATION

A characteristic of a tumor cell is continued replication under conditions in which normal cell growth would be retarded. Therefore it is possible that the surface membrane of transformed cells has properties that are more characteristic of growing cells and may reflect a less developed state than that of terminally differentiated non-growing cells. Fetal cell surfaces have a glycoprotein composition markedly different from adult cells and closely resembling that of transformed cells (52).

Quantitative differences are found in both glycoproteins and glycolipids from 3T3 and SV-3T3 (3T3 cells transformed with Simian virus) cells (53), with 3T3 cells containing 3 to 5 times as much sialic acid, N-acetylglucosamine, and N-acetylgalactosamine as SV-3T3 cells (54).

Many membrane alterations in glycoconjugates of transformants of Balb 3T3 cells have been described, but the only consistent alteration has been an enrichment in high molecular weight sialylglycopeptides (55). A review of glycopeptide changes upon malignant transformation has recently been published (56).

REGULATION OF GLYCOCONJUGATE SYNTHESIS BY GLYCOSYLTRANSFERASES

A clear focal point for anabolic regulation is with the biosynthetic enzymes themselves. With protein glycosylation, several points of regulation are possible. The O-glycosidic oligosaccharides may be regulated simply by the availability of transferases and/or substrates. Regulation of N-glycosidic glycosylation has more possible control points. Alterations in the dolichol pyrophosphate intermediate may result in complete absence of N-glycosidic linked carbohydrates, while regulation at the processing steps will determine the type of oligosaccharide formed (complex vs. high mannose). Addition of terminal carbohydrates as an important control point has been demonstrated by the role of terminal sugars in blood group

specificity. Considerable variation in terminal glycosylation has been shown to exist, as described below. The meaning of this diversity is not yet clear.

Terminal Glycosylation as a Regulatory Point

As mentioned previously, the nonreducing termini of the oligosaccharides of mammalian glycoproteins contain N-acetyglucosamine, galactose, fucose, and sialic acid. A multiplicity of linkages is possible and experimental evidence implies a distinct glycosyltransferase for each separate linkage. The structures which can be synthesized are dictated by the substrate specificities of the individual glycosyltransferases which are present. It has been shown that a number of the terminal transferases are mutually exclusive, with product formation being determined by the order in which they act (57).

Diversity of Terminal Structures

The terminal carbohydrates of bovine cold insoluble globulin (CIG) have several structurally unique features compared to many published structures for asparagine linked carbohydrates (58). The occurrence of NeuAc alpha 2-4 Gal is unusual in that most of the complex type sugar chains reported sialic acids are linked at the C-6 positions of the terminal galactoses. Human chorionic gonadotropin has been reported to contain sialic acid residues linked at the C-3 position of the penultimate galactose residue (59). This

diversity of the sialyl linkages on the complex type sugar chains may indicate that the specific terminal transferases act as regulatory signals. A Gal beta 1-3 GlcNac group is found in bovine CIG (58). This grouping has also been found in the sugar residues of bovine prothrombin (60) and the mucin type sugar chains of glycoproteins (61). Takasaki (62) has pointed out that all the N-acetylglucosamine residues of this grouping are sialylated at the C-6 position. A sequential relationship between the sialyl and galactosyltransferase probably exists.

The linkages observed in mammalian glycoproteins require at least 5 sialyltransferases and three fucosyltransferases (63). Oligosaccharide biosynthesis "in vivo" is regulated both by the glycosyltransferases available and the order in which they act (63). In the case of several sialyltransferases, structural features beyond the terminal N-acetylglucosamine units are recognized as preferred sialylation can occur on different branch chains (64). In the blood group substances the nature of the terminal saccharide is responsible for the immunological specificity. For example, the difference between blood groups A and B resides in the terminal D-galactose or D-N-acetylgalactosamine, and thus the determinant of specificity will reside in the regulation of the appropriate glycosyltransferase used for the terminal carbohydrate residue. Regulation of oligosaccharide synthesis has been studied by using purified glycosyltransferases, identifying

the products formed, and studying how the prior action of a particular glycosyltransferase will inhibit the action of another (57).

Fibronectin contains 4-5 percent carbohydrate of the complex-type linked via asparagine residues. Differences in carbohydrate structure exist between species with sialic acid linked to C3 of galactose in hamster fibroblasts (65), while the linkage is to the C4 or C6 of the penultimate galactose in human fibronectin (66). Different sugar chains of bovine fibronectin have been found to contain both beta 1-3 and beta 1-4 linked galactose (67).

Of at least 3 galactosyltransferases required in mammalian glycoprotein synthesis two have been purified (both soluble): one from milk, beta-N-acetylglucosaminide beta 1-4 galactosyltransferase (68), and the other from human plasma, designated (fucosyl alpha 1-2) galactoside alpha 1-3 galactosyltransferase Gal alpha 1-3 (Fucose alpha 1-2) Gal beta (63).

Penultimate galactose residues are beta-linked in most glycoproteins. Examples of alpha-linked galactose are earthworm cuticle collagen (69) and the blood group substances (70). The existence of alpha-linked galactose has also been demonstrated in bovine prothrombin, and interestingly, both anomeric forms of galactose are present on similar oligosaccharide chains (71).

The penultimate galactose of glycoproteins serves as a recognition signal in the clearance of serum proteins in mammals. In addition, the only tumor associated glycosyltransferase defined in tissue culture systems has been a galactosyltransferase (72). For these reasons, galactosyltransferases, in constrast to other glycosyltransferases, may deserve special recognition.

Alterations of Glycosyltransferases Upon Cellular Transformations

Cell Lines as Models

Cultured cell systems can be utilized as manipulatable models for studies that would be difficult or impossible to do "in situ". These systems can be exposed to hormones or other chemical agents and their response to this singular agent can be studied without complications arising from other cell or tissue types. Cell systems can easily be transformed by mutagenic or viral agents and biochemical alterations can be more easily monitored in these *in vitro* models.

In lectin-resistant cell lines, the cells have lost the ability to bind certain lectins and thus can withstand lectin toxicity. In some cases a specific glycosyltransferase responsible for a specific carbohydrate residue is deficient and altered surface glycoconjugates result. These lectin-selected mutants have been utilized to study the involvement of surface glycoconjugates in

recognition processes.

A specific wheat germ agglutinin (WGA) lectin-resistant CHO cell line has been utilized in studies described under results. WGA binds in a specific fashion to certain GlcNac, GalNac, and NANA residues in glycoconjugates. Several classes of NANA residues are present at the CHO cell surface, not all of which are involved in WGA binding (73). The particular cell line utilized in these studies possesses a defect in sialylation of surface glycoproteins (Pamela Stanley - unpublished data). Unlike the parental cell line, this mutant line shows extensive labeling of surface glycoprotein by treatment with galactose oxidase: [tritiated]borohydride without prior treatment with neuraminidase.

A similar cell line resistant to the cytotoxicity of the phytohemagglutinin from Phaseolus vulgaris has been shown to be deficient in a specific glycoprotein N-acetylglucosaminyltransferase activity (74).

Glycolipid Glycosyltransferases - When cells in culture are transformed by oncogenic viruses, alterations in glycolipid composition may occur at the level of the complex oligosaccharide chains. Reduced activity of N-acetylgalactosaminyltransferase has been reported in Swiss 3T3 cells infected with Murine Sarcoma virus (75). Mouse cells transformed by the RNA virus Kirsten sarcoma have lost UDP-Gal: Gm2 galactosyltransferase activity (76). This same galactosyltrans-

ferase is blocked in BALB/3T3 mouse embryo cells treated by X-irradiation and the chemical carcinogens methylcholanthrene and benzophyrene (77). In addition the low levels of galactosyltransferase activity in the transformed cells had kinetic properties indistinguishable from those in normal cells (77). Other ganglioside glycosyltransferases were unaffected, i. e. an N-acetylgalactosaminyltransferase and a sialyltransferase. Mixing experiments did not reveal the presence of any inhibitor of galactosyltransferase activity in these transformed cells. Nucleotide pyrophosphatase activity was unchanged between normal and transformed cells in this study. Other investigators have observed a decrease in sugar nucleotide pyrophosphatase activity in hamster cells after transformation (78).

Glycoprotein Glycosyltransferases

A clone of Chinese hamster ovary (CHO) cells, deficient in lectin binding sites, was found to have a selective deficiency of UDP-N-acetylglucosamine: glycoprotein N-acetylglucosaminyltransferase activity (79). Sialyl- and galactosyl-transferase activities were similar in normal and clone cells. This line shows a slightly altered cell morphology and weaker adhesion to tissue culture flasks (80).

Depression in a glycoprotein sialyltransferase (CMP-N-acetylneuraminate: D-galactosyl-glycoprotein N-acetylneuraltransferase, EC 2.4.99.1) has been reported in mouse cells transformed with a temperature-sensitive mutant of simian virus 40 grown at the restrictive temperature (81). Temperature sensitive alterations of glycosyltransferases have also been reported in cells transformed by Rous sarcoma virus (82).

Cancer Associated Alterations in Glycosyltransferase Activity

Differences in cell-surface-derived glycoproteins from normal and tumor derived cells have been noted by many investigators (reviewed in 56). Similarly, the presence of distinctive glycoproteins in the urine or serum of patients with cancer has led to the implication that these are derived from the tumor-cell surface (83,84).

Investigators have periodically reported increases in serum glycosyltranferase activity from patients with cancer. A general question is whether these tumor derived soluble glycosyltransferases are distinct from the membrane-bound forms. At least one investigator has proposed that soluble galactosyltransferases are produced by proteolytic cleavage of the membrane enzyme with both forms having similar kinetic and regulatory properties (85). The occurrence of cell-surface glycosyltransferases is still disputed but it should be noted that both increased and decreased cell-surface glycosyltransferase activities have been noted

in tumor cells (86,87). Increases in serum glycosyltransferase activities, presumbly sloughed off cancerous tissue, have been demonstrated, i. e. sialyltransferase (88,89) and fucosyltransferase (90,91).

Equivalent activity of total serum galactosyltransferase is found in normal controls and patients with cancer; however Podlsky et al. (92) has demonstrated the presence of an electrophoretically distinct form of galactosyltransferase in samples from cancer patients. The normal (GT-1) and cancer associated (GT-II) isoenzymes differ in molecular weight, kinetic properties, and carbohydrate content (92). Podolsky extended these studies to an animal model system in which hamsters were inoculated with polyoma transformed baby-hamster kidney cells (BHKpy). A serum isoenzyme GT-IIh could be solubilized from the resultant tumor but not from normal hamster tissue (93). In addition media from BHKpy cells grown in tissue culture contained a galactosyltransferase activity that co-electrophoresed with the GT-IIh found in tumor bearing animals. These findings suggest that the amount of GT-IIh detected was related to tumor mass. Total serum galactosyltransferase or total serum sialyltransferase fetuin acceptor activity was identical between control and tumor-bearing hamsters. These results constrast with the elevated levels of plasma sialyltransferase found in human cancer patients. (94).

Although a correlation between a specific galactosyltransferase isoenzyme and malignancy was found, a role for this isoenzyme in cellular behavior has not been shown. A distinct glycopeptide in sera and effusions of patients with extensive carcinoma has been detected (95). It was found to function as an acceptor for galactosyltransferase activity with a greater affinity for the cancer-associated galactosyltransferase (96). This acceptor caused a selective growth suppression of transformed cells in tissue culture (96) and contains 60-70 percent carbohydrate (96). The mode of growth suppression in transformed cells is unknown but it may be related to its ability to act as a substrate for tumor-associated galactosyltransferase.

Alterations of Glycosyltransferases in Development

Developmental changes require cellular recognition processes in that cells undergo specific orientations and rearrangements with respect to one another. Consequently many such processes may be regulated by cell surface glycoconjugates and/or cell surface glycosyltransferases. The adhesive specificity between embryonic cells and the migration of cells during development may be regulated by glycoconjugates (49).

A DSG-fetuin glycoprotein galactosyltranferase activity has been measured as a function of embryonic age in rat liver, lungs, and brain tissues (97). Enzyme activity was high at embryonic day 16 and then decreased with gestational

age. No molecular differences were found between embryonic and adult enzyme. Carlson et al. (98) measured two additional galactosyltranferase activities (acceptors were: 1) GlcNac and 2) desialized sheep submaxillary mucin) from embryonic rat pancreas, liver, and gut. The liver enzyme showed no specific trend, but the pancreatic transferases increased from 10- to 40-fold in specific activity from embryonic day 12 to birth.

A direct involvement of carbohydrate in differentiation is seen with Tunicamycin induced differentiation of human (HL-60) and murine (M1) myeloid leukemia cells in culture. After treatment with Tunicamycin morphological changes occurred in these cell lines that caused them to resemble mature myeloid cells. Fc receptors were induced in the M1 line (characteristic of differentiated cells). The implication of these observations is that glycosylation of cellular proteins plays a role in maintaining these myeloid leukemia cells in an undifferentiated state in culture (99).

Alterations of glycoproteins have been observed during the development of embryonic chick neural retinal cells (100). When a glycoprotein galactosyltransferase was examined in this system activity was found to decline as a function of embryonic age (101). When various glycosyltranferase activities were examined in liver cells of embryonic chicks, only a GDP-Man tranferase was found to increase with embryonic age (102). Den et al. (103) have reported that several glycosyltransferases undergo a

transition from soluble to membrane-bound forms during the development of the embryonic chicken brain.

In the slime mold, Dictyostelium discoideum, a large molecular weight glycoprotein is induced 12 hours after initiation of differentiation (104). Other studies with this system have implied that the carbohydrate moieties play roles as signals in cellular recognition phenomena (105).

A model system to study cell-cell aggregation phenomena is the sponge cell system. An aggregation factor purified from the sponge Geodia cydonium was found to contain as subunits: sialyltransferase (106), glucuronosyltransferase (107), and galactosyltransferase (107). A model relating these glycosyltransferases to a mechanism of adhesion has been proposed by Muller et al. (108).

An interesting model system for the study of differentiation dependent biochemical alterations is the epithelial cells of the villi of the small intestine. Epithelial cells at the base of the villus are the least differentiated. As the cells move up the villus they become progressively more differentiated until they reach the distal end of the villus where they slough off. Alterations of cellular adhesion factors clearly occur as the mature villi cells are more easily dissociated than the crypt cells. Studies by Weiser (109) with isolated villi cells found several alterations in enzymatic activity as the cells undergo differentiation. Incorporation of labeled sugars into glycoproteins increases as differentiation occurs with

maximal incorporation by the fully differentiated cells at the villus tip. Crypt cells have higher endogenous glycosyltransferase activity for transfer of fucose, mannose, N-acetylglucosamine, galactose, and glucose than the tip cells. Only CMP-sialic acid transferase activity (endogenous) is elevated in tip cells as opposed to the crypt cells. Since endogenous activity requires the "in situ" presence of transferase acceptors, an apparant increase in completed glycoslated products occurs as the cells differentiate and move towards the tip. The elevated CMP-sialic acid transferase in the tip cells may imply a regulatory point for terminal sialic acid.

Glycoproteins have frequently been reported to be altered upon transformation and have been correlated with cellular differentiation. These alterations are usually associated with altered synthesis patterns rather than glycosidic activities (110).

Rat Pancreas as a Developmental Model

Embryonic development is a complex series of temporally related events that starts with common genetic precursors and leads to a phenotypically diverse cellular architecture. The pancreas has served as a developmental model of differentiation at three distinct levels, which are : 1) histogenesis, in which endocrine, exocrine, and duct cells orient themselves into their organ specific morphologies, 2) cytodifferentiation, in which cellular differentiation

occurs, and 3) biochemical differentiation which involves the synthesis of cell specific structual and enzymatic proteins. Extensive information has been documented on the morphology and biochemistry of this organ during development (111,112) and consequently it is well suited as a model system for differentiation.

Since altered levels of various galactosyltranferases are known to occur during pancreatic development (98), it was decided to extend these observations in order to more fully elucidate the role of glycosylation in development.

Glucocorticoids have been shown to modulate the "in vitro" development of the embryonic rat pancreas (113), and similar steroids alter glycosyltransferase activities (114) in other organ systems. Consequently, a correlation may exist between the endocrine system and the level of glycosyltransferases.

Embryonic Differentiation - The pancreatic divesticulum emerges from the gut at 11 days of gestation in the rat and concurrently mesodermal cells condense around it to form the pancreatic bud. Low but significant levels of exocrine proteins are detectable at this early stage. Rapid cellular proliferation and morphogenesis follows with acinar structures becoming apparent at about 16 days. Levels of exocrine proteins do not rise significantly at this stage, which is termed the "protodifferentiated state" (115). After 16 days a large increase in the rough endoplasmic

reticulum occurs and distinct zymogen granules appear. The cells have now acquired the structural characteristics of mature cells. This secondary transition leads to the "differentiated state". At day 17 or 18 of gestation zymogen granules have accumulated near the apical surface and cytodifferentiation is complete.

An important technique in the developmental study of the pancreas was the development of the "in vitro" culture of pancreatic rudiments. It was found that under appropiate culture conditions pancreatic rudiments synthesize exocrine proteins at the same levels found in intact embryos of the equivalent age. This culture system has allowed controlled studies on the underlying mechanisms of regulation of the development of this organ. Rutter et al. (116) has demonstrated that the accumulation of digestive enzymes was dependent on the presence of mesenchymal tissue and that the factor involved could exert its effect without physical contact.

The thymidine analogue, 5-bromodeoxyuridine (BrdUrd) has been shown to exert a selective inhibition on cytodifferentiation of the embryonic rat pancreas cultured "in vitro" (117). Rudiments grown with BrdUrd appear to be normal but do not contain zymogen granules even though protein, RNA, and DNA synthesis occur at normal rates. Synthesis and accumulation of exocrine proteins is selectively inhibited by BrdUrd. BrdUrd does not inhibit the glycoprotein galactosyltrasferase to ovine submaxillary

mucin and does not inhibit the synthesis of proteins labeled with tritiated glucosamine (117). In addition DaGue (118) has shown that 5-BrdUrd has no effect on zymogen granule membrane-like glycoprotein synthesis. A general mechanism of BrdUrd action may involve altered binding of regulatory proteins to BrdUrd-containing DNA (117).

Embryonic pancreatic rudiments grown in culture incorporate radioactive L-fucose into a large number of glycoproteins some of which are induced during pancreatic differentiation (118). Walther (117) has reported that 5-BrdUrd, which prevents the "in vitro" secondary transition in developing exocrine pancreas, does not inhibit the synthesis of glycoproteins. Furthermore DaGue (118) found that glycoprotein profiles of SDS-polyacrylamide gels of tritiated glucosamine labeled control and 5-BrdUrd treated cultured rudiments were identical. The number of glycoproteins induced during pancreatic differentiation was found to be from five to seven depending on the sugar used to label the protein (118). These all occurred during the secondary transition period.

Glycosyltransferases as a Regulatory Mechanism in Protein Secretion

After synthesis on ribosomes, specific proteins may be transported across or integrated into distinct cellular membranes. Then subpopulations of these proteins need to be routed to other intracellular membranes or secreted. The role of the oligosaccharide moiety as a routing signal has

proved attractive. The information carried in the oligosaccharide could easily account for directing the protein to various points.

The role of glycosyltransferases in secretory cells is unclear. Although many secretory proteins are glycosylated, the pancreatic secretory proteins for the most part are not. Pancreatic secretory proteins are stored in zymogen granules and the purified zymogen granule membrane contains characteristic glycoproteins (119) which may, concerted with the action of specific glycosyltransferases, play important roles in secretion. Additional studies with rabbit antisera against highly purified zymogen granule membranes support the involvement of a galactosyltranferase with zymogen granule glycoprotein formation (120).

Rat Pancreas as a Secretory Model

The mammalian pancreas is a structually heterogeneous organ containing two major classes of cells: the beta-cells, which are primarily of endocrine function and the alpha-cells, which are exocrine and function in the synthesis and secretion of digestive zymogens. All subsequent discussions will be concerned only with the pancreatic acinar cells.

The rat pancreas has been chosen as a system in which to study the role of glycoprotein galactosyltransferase activity in secretion. Previous work by Ronzio (121) has partially characterized a system with respect to

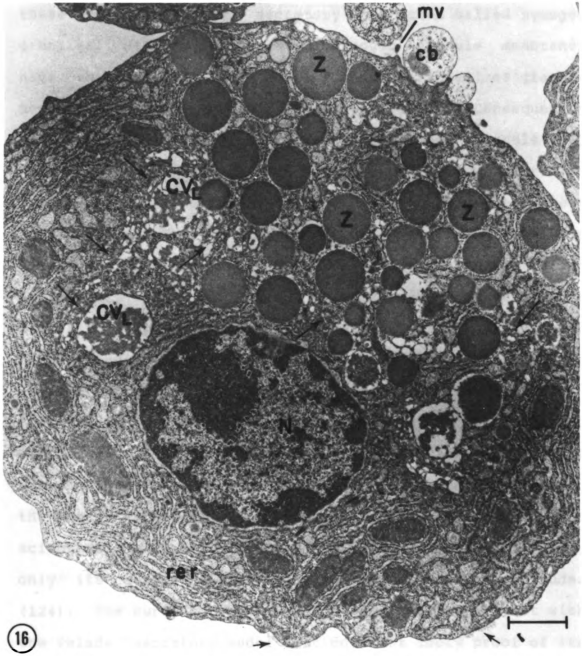
lycosyltransferase activity.

Secretory Process - The exocrine cells of the pancreas have provided an excellent model for studies of protein synthesis, segregation, intracellular transport, concentration, and discharge. Its role as an efficient protein synthesizer and secretor has led to its being utilized as a model cell for the study of such phenomena by numerous investigators. An electron micrograph of an isolated pancreatic acinar cell is shown in Figure 3. The sequence of events leading to secretion have been delineated through pulse-chase experiments utilizing radioactive amino acids followed by autoradiography and/or cell fractionation studies.

Proteins made for export are synthesized on polysomes attached to the rough endoplasmic reticulum (RER). The newly made secretory proteins are then segregated into the cisternal spaces of the RER. From the RER the proteins are transported to the transitional elements of the Golgi system, then to the small peripheral vesicles on the cis side of the Golgi complex and finally to the condensing vacoles. This intracellular transport requires energy and in the absence of ATP synthesis, the secretory proteins remain in the RER. The exact molecular nature of these steps remain to be elucidated. After reaching the condensing vacoles an increase in density occurs (as noted by electron microscopy) and the result is the conversion of

Figure 3. Transmission Electron Micrograph of a Dispersed Pancreatic Exocrine Cell

Transmission electron microscopy (x14,000) of this pancreatic exocrine cell demonstrates the complex architecture of this cell type. cb- cytoplastic blob, CV_L - loose condensing vaculoes, arrows- Golgi elements, Z- zymogen granule, marker- 1 μm (J. Cell Biol. (1974) 63, 1049)



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these vacoles to the secretory particles called zymogen granules. Studies of isolated zymogen granule membranes have shown that a major glycoprotein characterizes granule membranes of the mammalian pancreas (122). Consequently distinct topographical features of the zymogen granule (ZG) membrane are present which may play roles in concentration or secretion of the zymogens. The final step in the process is discharge. The ZGs discharge their contents into the glandular lumena thorough exocytosis. This membrane fusion occurs only with the apical plasmalemma. Secretion is stimulated upon hormonal action at the basal cell surface. An outstanding current problem is the coupling of secretion to this spacially distant hormonal stimulation.

The above model of pancreatic secretion proposed by Palade and his collaborators (123) has achieved general acceptance and in part for these studies Palade was awarded the Nobel Prize. The acceptance of a paradigm by the scientific community by no means implies its correctness, only its acceptability to current scientific attitudes (124). The current experimental evidence is consistent with the Palade "secretory model" but does not imply proof of its existence nor are alternative interpretations of the data excluded. In addition, several experimental observations are not easily accounted for by the current paradigm.

Consequently an alternative model of secretion has been proposed by S. S. Rothman (125). Palade's theory assumes complete sequestration of secretory proteins from the

cytoplasm from the point of synthesis to secretion of zymogen granules by exocytosis. Rothman's model, i.e. an "equilibrium system", proposes that the cytoplasm of the cell acts as both a mixing chamber for digestive enzymes as well as a precursor pool for secretion. Several lines of evidence are consistent with this theory. Digestive enzymes in zymogen granules have been reported to be in equilibrium with enzyme in the surrounding medium across the granule membrane (126,127). In suspended tissue slices, labeled enzyme equilibrates across the cell membrane, through the cytoplasm, and into the zymogen granules (126,127). Upon cholinergic stimulus a depression of the specific radioactivity of secreted protein is found. This is contrary to what is expected from Palade's model but is consistent with the hypothesis that zymogen granules discharge their contents into the cytoplasm prior to secretion across the cell membrane (128). The two models are not mutually exclusive, however, and both processes may be occuring concurrently.

Glycoproteins are important constituents in all membrane subfractions of the exocrine pancreas. The zymogen granule membranes are high in protein bound galactose and fucose and have a mass ratio of carbohydrate to protein of 0.44 (122).

Zymogen granule membranes contain 3 major glycoproteins, designated GP-1 (MW 120,000), GP-2 (MW 74,000) and GP-3 (MW 52,000), which become labeled with

tritiated glucosamine (122). Upon immunoprecipitation with antibodies made against purified zymogen granule membrane a labeled glucosamine-labeled glycoprotein of the same eletrophoretic characteristics of GP-2 is detected in the smooth microsomal Golgi-enriched fraction. GP-2 has similarly been immunologically detected in both the "protodifferentiated" and the "differentiated" pancreas (Ronzio, unpublished observations).

Present evidence implies that each zymogen granule contains the same complement of digestive enzymes and that sub-populations of zymogen granules do not exist (129). Therefore secretory transport has no selectivity and regulation of enzyme concentration must be derived from selective synthesis. A specific stimulus can alter the rate of secretion of one enzyme relative to another; however the molecular basis of this phenomenon is not clear. Ronzio and Mohrlok (120) have described a Golgi membrane associated galactosyltransferase involved in the formation of zymogen granule glycoproteins.

Alterations of Glycosyltransferases by Chemical Agents

Butyrate - The addition of sodium butyrate to the media of a variety of cell lines causes biochemical and morphological changes. In several cases the morphological changes induced by butyrate can be blocked by the addition of dibutyryl-cAMP (130,131,132). Butyrate has also been found to induce a glycolipid glycosyltransferase in HeLa (133) and KB (134)

cells. cAMP has also been found to oppose the sialyltransferase induction by butyrate in HeLa cells. Cell surface carbohydrates have been found to vary in tissue culture cells when they are grown in the presence of butyrate (135). The mechanism of the butyrate effect is unknown but recent observations indicate it may be related, in KB cells, to a blocking of the cell cycle at the G1 stage (136).

Phorbol Ester Tumor Promoters - A tumor promoter is a type of co-carcinogen that enhances tumor formation when administered after initiating action by a carcinogen. In the two-stage model of carcinogenesis a subcarcinogenic dose of carcinogen is applied to mouse skin and this is followed by repeated applications of a promoter. If the promoter treatment is continued carcinomas develop. The initiating event is irreversible and probably occurs at a mutagenic level. On the other hand the effects of the promoter are reversible, are not mutagenic and may involve interruptions of normal intercelluar communication (137).

Studies directed towards understanding the mechanism of tumor promotion are increasingly utilizing cultured cell systems as models. When phorbl ester tumor promoters are added to cells in culture a diversity of effects have been noted. Alterations have been observed in DNA and RNA synthesis, phospholipid metabolism, prostaglandin synthesis, nutrient uptake, cellular morphology, cell division, terminal differentiation, etc.

Driedger and Blumberg (138) have described three generalizations of their biological activities: 1) normal cells will assume a transformed phenotype upon exposure to phorbol esters (139), 2) phorbol esters cause superexpression of transformation-sensitive properties (140), and 3) the phorbol esters modify differentiation and differentiated cell functions (141). These phorbol ester induced alterations have all been previously linked to involvement with glycoconjugates, and consequently their effect on several glycosyltransferases has been examined in this investigation.

Miscellaneous Agents

Surface glycoconjugates are also altered in cells grown in tissue culture by the agents cyclic AMP (142) or ethidium bromide (143).

Turpentine injection is rats causes a two fold increase in microsomal galactosyltransferase specific activity in the liver. This increased activity was due to a proliferation of Golgi membrane rather than a specific induction of the glycosyltransferase (144). An inhibitory effect by cycloheximide on galactosyltransferase activity in rat liver Golgi membranes has been shown but the conclusions were that this effect is secondary to the primary action on the membrane system (145).

MATERIALS AND METHODS

MATERIALS

Electrophoresis Reagents

Acrylamide (99 percent)	Bio-Rad Laboratories, Richmond,Ca
N,N´-Methylenebisacrylamide	Bio-Rad Laboratories, Richmond,Ca
N,N,N´,N´-Tetramethyl-ethylenediamine (TEMED)	Bio-Rad Laboratories, Richmond,Ca
Ammonium Persulfate	Fisher Scien. Co., Fair Lawn,NJ
Sodium Dodecyl Sulfate	Pierce Chemical Co., Rockford,Il
Bromphenol Blue	Nutritional Biochemical Corp., Cleveland,Oh
Coomassie Brilliant Blue R	Sigma Chemical Co., St. Louis,Mo
Beta-Mercaptoethanol	Sigma Chemical Co., St. Louis,Mo
Basic Fuchsin	Sigma Chemical Co., St. Louis,Mo

Radiochemicals

[Galactose Carbon-14]Uridine Diphosphate Galactose	New England Nuclear,Boston,Ma
[Gamma Phosphate-32]Adenosine 5´-Triphosphate	New England Nuclear,Boston,Ma
[Carbon-14]Galactose-1-Phosphate	New England Nuclear,Boston,Ma
[Carbon-14]Galactose	New England Nuclear,Boston,Ma
[Tritiated]Thymidine	New England Nuclear,Boston,Ma

Liquid Scintillation Counting

Triton X-100	Research Products Internat. Corp.,Elk Grove Village,Il
Dimethyl-POPOP (1,4- is[2-(4-Methyl-5-Phenyloxazolyl)]-Benzene	Research Products Internat. Corp.,Elk Grove Village,Il
POP (2,5-Diphenyloxazole)	Research Products Internat. Corp.,Elk Grove Village,Il

Tissue Sources

Rats (Sprague-Dawley)	Spartan Research,Haslett,Mi

Cell Lines

Hamster Fibroblast-Normal (Nil-8)	Dr. P. W. Robbins,MIT, Cambridge,Ma
Hamster Fibroblast Transformed with Hamster Sarcoma Virus (Nil-8HSV)	Dr. P. W. Robbins,MIT, Cambridge,Ma
Human Epithelial Carcinoma (KB)	American Type Culture Collection,Rockville,Md
Chinese Hamster Ovary (CHO)	Dr. Pamela Stanley,Albert Einstein,New York,NY
Hamster Fibroblasts (V79's)	Dr. James Trosko,MSU, East Lansing,Mi

Tissue Culture

Minimal Essential Medium (MEM)	Grand Island Biological Co., Grand Island,NY
Dulbecco's Modified Eagle Medium (DMEM)	Grand Island Biological Co., Grand Island,NY
Fetal Calf Serum	Grand Island Biological Co., Grand Island,NY
Calf Serum	Grand Island Biological Co., Grand Island,NY
Earle's Balanced Salt Solution	Grand Island Biological Co., Grand Island,NY
Penicillin-G	Sigma Chemical Co., St. Louis,Mo
Streptomycin Sulfate	Sigma Chemical Co., St. Louis,Mo

Miscellaneous

Fetuin (Spiro Method)	Grand Island Biological Co., Grand Island,NY
Bovine Submaxillary Mucin	Sigma Chemical Co., St. Louis,Mo
Uridine Diphosphate Galactose (UDP-Gal)	Sigma Chemical Co., St. Louis,Mo
Adenosine Triphosphate (ATP)	Sigma Chemical Co., St. Louis,Mo
Adenosine Monophosphate (AMP)	Sigma Chemical Co., St. Louis,Mo

2-(N-Morpholino) Ethane Sulfonic Acid (MES)	United States Biochem. Corp., Cleveland,Oh
Orosomucoid	Dr. Don Carlson,Purdue University,Lafayette,In
Filter Discs (0.45uM)	Millipore Corp.,Bedford,Ma
Retinoic Acid	Sigma Chemical Co., St. Louis,Mo
Butyrate	Sigma Chemical Co., St. Louis,Mo
Epidermal Growth Factor (EGF)	Collaborative Research,Inc., Waltham,Ma
Mellitin	Dr. James Trosko,MSU, East Lansing,Mi
Phorbol Ester Tumor Promotors	Dr. James Trosko,MSU, East Lansing,Mi
Alpha-Lactalbumin	Sigma Chemical Co., St. Louis,Mo
Ampholytes	LKB Inc.,Chicago,Il
Bio-Gel P-200	Bio-Rad Laboratories, Richmond,Ca
Sephadex G-100	Sigma Chemical Co., St. Louis,Mo
Trichloroacetic Acid (TCA)	Fisher Scien. Co., Fair Lawn,NJ
Phosphotungstic Acid (PTA)	Sigma Chemical Co., St. Louis,Mo
Cacodylic Acid (Dimethylarsinic Acid)	Sigma Chemical Co., St. Louis,Mo
Dolichol Monophosphate	Sigma Chemical Co., St. Louis,Mo
Sodium Meta Periodate	G.F. Smith Chemical Co., Columbus,Oh
Blue Dextran	Pharmacia Fine Chemicals, Uppsala,Sweden
X-Ray Film	Eastman Kodak Co.,Rochester,NY

All additional chemicals and materials were reagent grade.

METHODS

Rat Pancreatic Tissue as a Galactosyltransferase Source

Dissection and Homogenization of Adult Rat Pancreas

Sprague-Dawley rats weighing 100 to 300 grams were stunned by a blow to the head, decapitated, and the pancreas immediately dissected and removed into a petri dish sitting in an ice bucket. The pancreatic tissue was cleaned of fat and connective tissue, minced in 0.3 M sucrose and homogenized with 10 strokes in a Potter-Elvehjem homogenizer. The homogenate was filtered through several layers of cheese cloth and centrifuged at 500 x g to sediment debris, nuclei, and unlysed cells. This supernatant fraction was labeled the "crude homogenate". Microsomes were prepared from this supernatant fraction by centrifigation at 100,000 x g for 1 hour (Beckman Model LS-50 preparative ultracentrifuge). The supernatant fraction was discarded and the microsomal pellet was removed and resuspended in a small volume of 0.3 M sucrose with several strokes of a Potter-Elvehjem homogenizer. This microsomal fraction was then assayed directly or stored frozen at -80 degrees C.

Dissection and Homogenization of Embryonic and Neonatal Rat Pancreas

Pregnant Sprague-Dawley females of appropriate gestation age were stunned by a blow to the head, decapitated, and the uterus dissected and placed in a petri dish in an ice bucket. Embryos were removed and placed in Earle's balanced salt solution. The pancreases were dissected in plastic petri dishes using iridectomy knives with the aid of a binocular microscope. Embryonic age was confirmed by comparing the age-specific embryonic characteristics described by Christie (146) with the dissected tissue. For homogenizaton of the pancreases the tissue was placed in a microfuge tube containing 0.100 ml of ice-cold phosphate buffered saline (PBS). The microfuge tube was taped to the bottom of a plastic tray and submerged in ice-water. A sonicator probe (Biosonik II, Bronwill Scientific, Rochester, NY) was then rubbed against the microfuge tube (setting #5 for 5 sec) to disrupt the tissue. The resulting homogenate was used directly in the studies performed. The extremely small quantity of tissue present did not allow additional fractionation of embryonic tissue to microsomes.

Neonates (day 1 is day of birth) were dissected with the aid of a dissecting scope and iridectomy knives and the pancreases pooled. Samples were homogenized by sonication or with a Potter-Elvehjem homogenizer in an identical manner as the adult tissue. Microsomes were prepared (when tissue

quantity permitted) as for adult tissue.

Subcellular Fractionation of Rat Pancreatic Tissue

The procedure of Jamieson and Palade (147) as modified by Ronzio (148) was used to fractionate rat pancreas rapidly with minimal degradation. Tissue was processed as described above to the 500 x g supernatant stage (crude homogenate). This supernatant fraction was centrifuged for 10 minutes at 1750 x g to collect the white zymogen granule pellet. The 1750 x g supernatant fraction was then centrifuged at 8500 x g for 15 min. The resulting pellet was called the mitochondrial fraction. The post-mitochondrial supernatant fraction was then centrifuged for 1 hour at 100,000 x g. The supernatant fraction was termed the soluble fraction and the resulting crude microsomal pellet was further fractionated into smooth and rough microsomal fractions. This crude microsomal pellet was suspended in a small volume of 0.3 M sucrose by means of a Potter-Elvehjem homogenizer and centrifuged for 195,000 x g for 1 hour on a continuous sucrose gradient (0.5-1.5M). Smooth microsomes banded near 0.70 M sucrose while the region at the bottom of the tube contained the rough microsomal fraction. Fractions were gently removed with a Pasteur Pipet and frozen at -80 degrees C until assayed.

Cultured Cells as a Galactosyltransferase Source

Growth Conditions

Cell lines were maintained in a humidified atmosphere of 5 percent carbon dioxide-95 percent air at 37 degrees C and were routinely subcultured upon reaching confluency with a 0.05 percent trypsin solution containing 0.02 percent ethylenediaminetetraacetic acid (EDTA). Cells were normally grown as monolayers in plastic tissue culture flasks (Corning Glass Works, Corning,NY). Several cell lines (human epithelial and chinese hamster ovary cells) were grown in suspension culture. Human Epithelial (KB) and hamster V79 cells were grown in minimum essential medium (MEM) containing calf serum (10 percent), penicillin (100 ug/ml), and streptomycin (100 units/ml). KB cells grown in suspension culture had calcium free medium. Hamster fibroblasts (Nil-8, and a transformed line, Nil-8HSV) were grown in Dulbecco's modified Eagles medium with fetal calf serum (10 percent), penicillin (100 ug/ml), and streptomycin (100 units/ml). CHO cells were grown in alpha modified Eagles medium (MEM plus ribonucleosides and deoxyribonucleosides) with calf serum (10 percent), penicillin (100 ug/ml), and streptomycin (100 units/ml).

Addition of Chemical Effectors

Cell lines were normally maintained until cell confluency reached 40-60 percent. At this time fresh media containing the appropriate chemical effector was added to the culture flasks. After an incubation period of 12-16 hours, the media was removed, the cells washed twice with PBS, and the cells harvested.

Synchronization of Growth

Log phase cells were synchronized in the early part of the late G1 period by exposure to two cycles of thymidine inhibition (149). Media containing 2 mM thymidine was placed on the cells for 24 hours. This media was then removed and the cells washed three times with $phosphate buffered saline (PBS) followed by the addition of fresh media. After 12 hours the media was removed, the cells were washed with PBS, and media containing 2mM thymidine was placed on the cells for 24 hours. After this second block the thymidine containing media was removed, the cells were washed in PBS, and fresh media put on the cells. Synchronization was confirmed by measuring radioactive thymidine incorporation into DNA.

DNA Labeling

DNA pulse-labeling was carried out by adding tritiated thymidine (52 mCi/umole; 0.1 uCi/ml) to control flasks at 60 minute time points after synchronization. After a 60 minute incubation with tritiated thymidine the cells were washed twice with ice cold PBS, incubated at 4 degrees C for 15 minutes in the presence of 10 ml of ice cold 5 percent trichloroacetic acid (TCA) and scraped from the flasks with a rubber policeman. The flasks were then rinsed with an additional 10 ml of 5 percent TCA and the precipitated material pelleted by centrifugation at 100 x g. Pellets were then washed twice with PBS and solubilized with 0.1 N NaOH overnight. Aliquots were assayed for protein (Lowry) and for radioactive incorporation of thymidine (by liquid scintillation counting).

Harvesting and Homogenization

Cells were removed for assays from tissue culture flasks by scraping with a rubber policeman or by a short incubation at 37 degrees C with 0.05 percent EDTA in PBS. A small amount of PBS was then added to the cells, the cells were suspended by swirling, and the suspended cells were then poured into centrifuge tubes. The tissue culture flasks were then rinsed with a second PBS wash. Cells were then centrifuged into pellets (500 x g), the pellets were washed with PBS, and then stored frozen at -80 degrees C.

Cells were disrupted by mild sonication or by means of a Potter-Elvehjem homogenizer as described previously for pancreatic tissue.

Galactosyltransferase Assays

Assays with Exogenous Protein Substrates

The assay mixture contained in a final volume of 0.05 ml : 0.25 M sodium N-morpholino-2-ethanesulfonate (MES), pH 6.7; 10 mM manganese chloride; 0.5 percent Triton X-100; 0.125 mg desialylated degalactosylated fetuin (DSG-fetuin); and 0.46 mM UDP-galactose (UDP-galactose was added to UDP-[carbon- 14] galactose (280 mCi/mmole)) to a final concentration of 0.46 mM (22,000 dpm). After addition of sample (1 to 50 ug protein) assays were incubated for 30 minutes at 37 degrees C. Reactions were terminated by the addition of 5 ml of ice cold 5 percent phosphotungstic acid in 0.1 N HCl. Precipitates were collected on Millipore filters (0.45 uM pore size), washed three times with 5 ml of the precipitation solution, washed with 2 ml of diethyl ether, placed in scintillation vials and dissolved in 1 percent sodium dodecyl sulfate-0.1 N NaOH. After neutralization with 1 N HCl, 10 ml of toluene-Triton X-100 scintillation fluid (147) was added and the samples counted by liquid scintillation spectrometry.

Assays Involving Endogenous Lipid Acceptors

Incorporation of [Carbon-14]galactose into endogenous lipid acceptors was determined by extracting the galactosyltransferase reaction mixture with chloroform-methanol (2:1, (v/v)). The lower phase was then dried in a scintillation vial, solubilized and counted.

Assays with Free Glucose and Free N-Acetylglucosamine as Substrates

The assay mixture contained in a final volume of 0.05 ml: 0.25 M sodium cacodylate buffer, pH 7.5; 10 mM manganese chloride; 50 mM glucose and/or 50 mM N-acetylglucosamine; 0.6 mM UDP-galactose (UDP-galactose was added to UDP-[carbon-14]galactose to a final concentration of 0.6 mM (5000 d.p.m.)); and 0.001 to 0.050 mg protein. Some assays contained in addition to the above 0.0075 mg of alpha-lactalbumin. After incubation for 30 minutes at 37 degrees C the assays were terminated by the addition of 0.5 ml of ice-cold water. The entire assay mixture was then passed through a Dowex 1 column (Cl minus, 8 percent, 100-200 mesh) made in a Pasteur Pipet. The column was washed with 1.5 ml of water and the elutant and wash collected into a scintillation vial. Scintillation fluid was added (toluene-Triton X-100 mix) and the samples counted by liquid scintillation spectrometry.

Preparation of Desialylzed and Degalactosylated Protein Acceptors

The method of Kim (150,151) was utilized to obtain protein acceptors free of the terminal N-acetylneuraminic acid and the penultimate galactose residues. The protein solution (10 - 20 mg/ml) was heated for 1 hour at 80 degrees C in 0.1 N sulfuric acid to remove N-acetylneuraminic acid. The solution was then neutralized with 0.1 N NaOH and dialyzed overnight against distilled water. The nondialyzable material was treated with 0.01 M sodium meta periodate in 0.05 M sodium acetate, pH 4.5, overnight at 4 degrees C in the dark. The reaction was stopped by the addition of glycerol and then dialyzed overnight against 4 changes of distilled water. The dialyzed material was incubated for 13 hours at 4 degrees C in a 0.15 M potassium tetraborate buffer, pH 8.0 containing 0.15 M sodium borohydride. After 13 hours the pH was adjusted to 5.0 with 2 N acetic acid and the sample was exhaustively dialyzed against distilled water. The dialysate was made 0.05 N in sulfuric acid, heated for 1 hour at 80 degrees C, neutralized with NaOH, dialyzed against distilled water and lyophilized. The lyophilized material was stored dessicated at -20 degrees C.

Physical Characterization of Microsomal Galactosyltransferase Activity

Isoelectric Focusing

Isoelectric focusing studies were performed in a sucrose density gradient column according to the procedure of Massey and Deal (152). Conventional polyacrylamide gel electrophoresis apparatus was utilized to carry out single tube isoelectric focusing experiments in sucrose density gradients. Microsomes were dissolved in 5 percent Triton X-100, spun at 100,000 x g for 1 hour and the supernatant fraction removed and added to both the light ampholyte solution (5 percent sucrose (w/v), 0.43 percent ampholytes) and to the heavy ampholyte solution (20 percent sucrose (w/v), 2 percent ampholytes). A gradient maker was then used to form 5.6 ml gradients in each column. Electrofocusing was conducted at 200 volts for 4 to 10 hours at 4 degrees C. Fractions were collected at the top of the column by forcing a 50 percent sucrose solution (w/v) into the bottom of the column with a 20 cc syringe equipped with a 22-gauge hypodermic needle. Fractions of 0.400 ml were collected, the pH of each was measured with a Beckman pH meter and 0.050 ml aliquots were assayed for galactosyltransferase activity (reaction mix modified from standard to allow for final assay volume of 0.100 ml).

SDS Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out using the system of Laemmli (153). The separating gel was 7 percent acrylamide and contained Tris-HCl (pH 8.8) and 0.1 percent SDS. The stacking gel was 3 percent acrylamide and contained Tris-HCl (pH 6.8) and 0.1 percent SDS. Polymerization was initiated by the addition of tetramethylethylenediamine and ammonium persulfate.

Samples for electrophoresis were prepared by adding the sample to an equal volume of protein buffer containing: 0.0625 M Tris-HCl (pH 6.8), glycerol 12 percent (v/v), 1.25 percent (w/v) SDS, 1 percent 2-mercaptoethanol, and 0.001 percent (w/v) bromphenol blue. The samples were heated in a boiling water bath for 2 to 3 minutes before applying to the gel. The buffer used in the top and bottom reservoirs consisted of 0.025 M Tris base, 0.19 M glycine, and 0.1 percent SDS (w/v) (pH 8.3). Electrophoresis was done in a Bio-Rad Model 220 slab gel electrophoresis unit at 25 mA per gel until the bromphenol blue had migrated within 1 to 2 cm of the bottom of the gel (6 to 8 hours). Protein standards used for molecular weight calibration were: Cytochrome C - 12,500 daltons (Boehringer Mannheim); Chymotrypsinogen - 25,000 daltons (Boehringer Mannheim); Egg Albumin - 45,000 daltons (Boehringer Mannheim); Bovine Albumin - 67,000 daltons (Boehringer Mannheim); Phosphorylase A - 94,000 daltons (Sigma); and Beta-Galactosidase - 130,000 daltons

(P-L Biochemicals).

After electrophoresis the gels were stained for protein in a solution of 0.1 percent Coomassie brilliant blue R in 10 percent (v/v) acetic acid (154). After staining overnight gels were destained by multiple washes with 10 percent (v/v) acetic acid. Gels were stained for carbohydrate using the periodic acid-Schiff reagent (155). It was imperative that all traces of SDS be removed from the gel by repeated washes with 10 percent acetic acid before the PAS stain was applied. The procedure of Fairbanks et al. was (156) used for the PAS staining process.

Molecular Exclusion Chromatography

Solubilized microsomes were run on a Bio-Gel P-200 (exclusion limit 200,000 daltons) column in order to determine if multiple peaks of activity could be detected. Bio-Gel P-200 (100-200 mesh) was hydrated in 0.125 M MES buffer plus 0.5 percent Triton X-100 and poured into a column measuring 0.9 x 80.0 cm. Microsomes were homogenized in running buffer, centrifuged for 1 hour at 100,000 x g, and the supernatant fraction was applied to the column. Fractions of 0.250 ml were collected and 0.50 ml aliquots from each were assayed for galactosyltransferase activity. The column was calibrated with Blue Dextran (Vo) and potassium ferricyanide (Ve).

Pyrophosphatase Assays

Pyrophosphatase activity was determined with the identical assay mix for galactosyltransferase except for deletion of the exogenous acceptor. The reaction was terminated by the addition of 0.100 ml ethanol and 0.050 ml of 5 percent acetic acid (v/v) . Insoluble material was removed by centrifugation at 10,000 x g for 2 minutes with a microfuge (Model 6-811, Coleman Instruments Inc, Maywood, Ill). The supernatant fraction was run on a descending paper chromatographic system as described under miscellaneous methods. Radioactive standards (all carbon-14) galactose, galactose-1-phosphate and UDP-galactose were run to calibrate the system. Radioactivity was quantitated in each peak by cutting the chromatograms into pieces and counting them by liquid scintillation spectrometry.

Phosphorylation Assays

Purification of Membrane Fractions

Membrane fractions were prepared from CHO and KB cells by the procedure of Thom et al. (157). Cells were lysed by dilution into hypotonic borate/EDTA buffer, pH 10.2. After removal of debris and precipitated material by low speed centrifugation (450 x g for 10 minutes), a crude membrane fraction was obtained by centrifugation of the supernatant for 12,000 x g for 30 minutes. The pelleted material was

resuspended, layered over 35 percent (w/v) sucrose and centrifuged at 40,000 x g for 45 minutes in a swinging bucket rotor. The material layering at the sucrose-buffer interface was removed, suspended in 10 mM Hepes, pH 7.4, and recentrifuged at 100,000 x g for 30 minutes. The pellet was then resuspended in Hepes buffer and stored at -80 degrees C until assayed.

Assay Conditions

The reaction mixture contained: Hepes buffer, 20mM, pH 7.4; manganese chloride, 1mM, gamma 32p, 600,000 cpm; ATP, 15 uM; bovine serum albumin, 7.5 ug; and membrane protein, 10-60 ug in a final volume of 00.60 ml. The standard reaction was initiated by the addition of labeled ATP and after incubation at 4 degrees C for 4 minutes, was terminated by the addition of ice cold 10 percent trichloroacetic acid (TCA) containing 0.01 M sodium pyrophosphate (NaPP). The reaction mixture was placed on Millipore filters (pore size 0.45 um), washed extensively with 10 percent TCA-0.01 M NaPP, washed with diethyl ether, dried and counted by liquid scintillation spectrometry. Promotors were added to the reaction mix before addition of labeled ATP and incubated at 37 degrees C for 5 minutes. After the 37 degrees C incubation, the assay tubes were cooled to 4 degrees C and the assay initiated with the addition of labeled ATP.

Miscellaneous Methods

Paper Chromatography

Descending paper chromatography was used to determine the rate of breakdown of UDP-galactose in the pyrophosphatase assay as well as to check the purity of UDP-galactose used in the galactosyltransferase assays. Whatmann 3 MM paper was cut into strips 3 x 50 cm and samples were run with a solvent system of ethanol (75 ml)-sodium acetate buffer (30 ml-1M, pH3.8) (158) for 17-18 hours. Radioactive standards, UDP-galactose, galactose-1-phosphate, and galactose (all New England Nuclear) were run on duplicate strips. After drying, the paper strips were scanned for radioactivity with a radiochromatogram scanner (Packard Model 7201) and the areas corresponding to radioactive peaks were cut out, placed in scintillation vials, and the radioactivity quantitated by liquid scintillation counting.

Lowry Protein Assay

Protein was determined by the method of Lowry *et al.* (159) using bovine serum albumin as standards.

Purification of Alphalactalbumin

Alpha lactalbumin was obtained from Sigma (Grade II, 90 percent pure) and purified by exclusion chromatography on Sephadex G-100. The sample (500 mg) was applied in ammonium

carbonate buffer to a column (2.5 x 90 cm) previously calibrated with Blue Dextran (Vo) and potassium ferricynide (Ve). Absorbance of column fractions was monitored at 295 nm and the major peak fractions were pooled, dialyzed against distilled water and lyophilyzed.

Liquid Scintillation Counting

Radioactive samples were counted on a Beckman LS-150 or a Hewlett Packard 2405 scintillation counter. The scintillation cocktail used was that of Patterson and Green (160) and contained one part Triton X-100 to two parts toluene (0.015 percent 1,4-bis[2-(4-Methyl-5-phenyloxazolyl)]-benzene (POPOP) and 0.82 percent 2,5-Diphenyloxazole (PPO)). Samples containing up to one ml of water could be used with ten ml of this cocktail.

Autoradiography

Radioactively labeled proteins were run on polacrylamide slab gels. The gels were dried on a gel drier (Bio-Rad Laboratories, Model 224) and autoradiography was performed at -80 degrees C with X-Omat R film (Kodak) for exposure periods of 1-3 weeks.

Electron Microscopy

Transmission electron microscopy was performed with a Philips Model 300. Membrane samples were negatively stained with 2 percent ammonium molybdate.

RESULTS

Requirements of UDP-Galactosyltransferase from Pancreatic Rat Microsomes

The assay for membrane-bound UDP-galactose: glycoprotein galactosyltransferase is described in Methods. The transfer reaction and the competing UDP-galactose pyrophosphatase activity are outlined in Figure 4. Requirements for the transfer of galactose to desialized and degalactosylated fetuin (DSG-fetuin) are described in Table 1.

Cation Requirements - The reaction is dependent upon manganese chloride, although partial activity can be restored when magnesium chloride (40 mM) is substituted for manganese chloride. The optimal manganese chloride concentration for enzymatic activity was found to occur near 10 mM as shown in Figure 5. Addition of EDTA (5 mM) to the complete reaction mixture reduces activity by approximately 80 percent. When manganese chloride was replaced with calcium chloride, zinc chloride, barium chloride, cobalt chloride, zinc sulfate, or mercury chloride (all at 40 mM) only trace levels of galactosyltransferase activity could be detected.

Effect of Detergent and Dithiothreitol - When the assay was run in the absence of detergent, activity was reduced to near endogenous levels. Maximum activity was found to occur in the presence of 0.5 percent Triton X-100. Other

Figure 4. UDP-Galactose Glycoprotein: Galactosyltransferase and UDP-Galactose Pyrophosphatase Reactions

UDP-galactose is utilized as a substrate for the reactions outlined in the figure. The glycoprotein galactosyltransferase transfers galactose from UDP-Gal to the N-acetylglucosamine residue at the non-reducing termini of a oligosaccharide attached to a protein though a asparagine residue. A competing pyrophosphatase reaction cleaves UDP-Gal to galactose-1-phosphate which is then further degraded to galactose and inorganic phosphate.

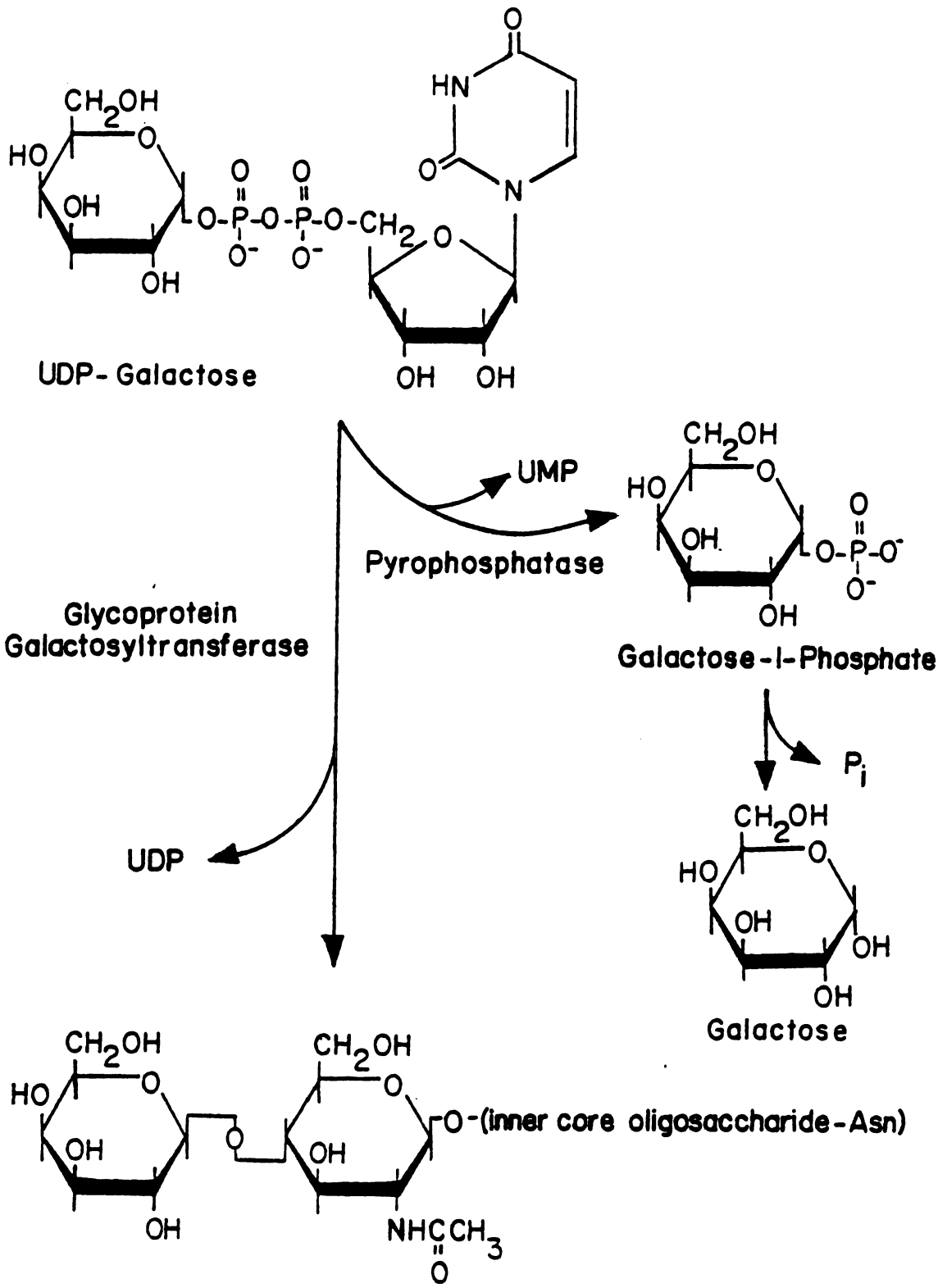

UDP- Galactose
UMP
Pyrophosphatase
Glycoprotein
Galactosyltransferase
Galactose-1-Phosphate
P_i
UDP
Galactose
O-(inner core oligosaccharide-Asn)

Table 1.

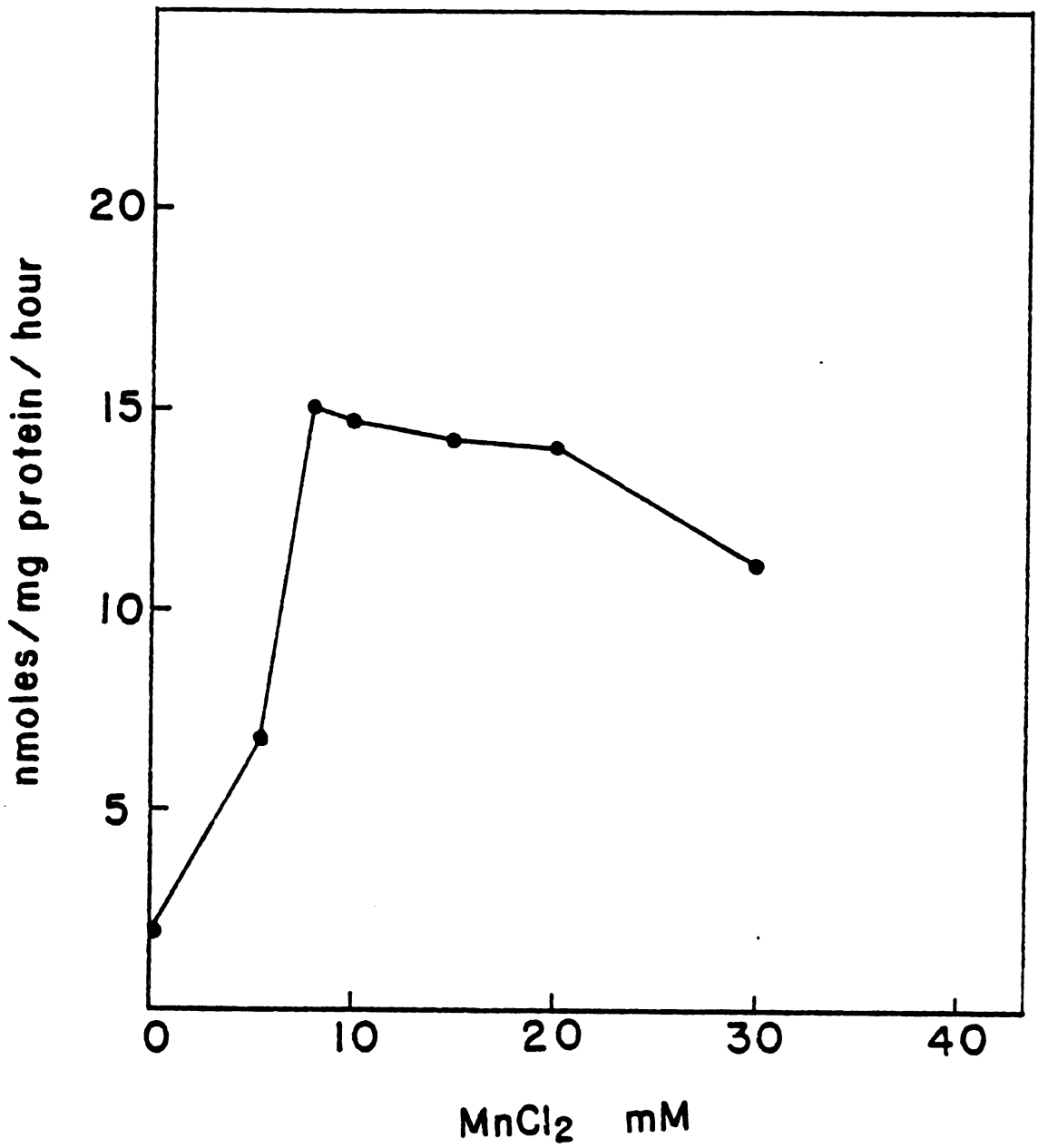
Requirements for the Incorporation of [^{14}C]Galactose into Desialylated Degalactosylated Fetuin by Pancreatic Microsomes

Modification of Reaction Mix	Activity(%)
Complete[a]	100
Minus $MnCl_2$	5
Minus $MnCl_2$ plus $MgCl_2$	25
Plus EDTA(5mM)	20
Minus Triton-100	5
Minus DSG-Fetuin	10
Plus Dithiothreitol(1mM)	100
Plus AMP(3.4mM)	100
Boiled Enzyme	5
Incubation at 4°C(one half hour)	15

[a]Assay for galactosyltransferase is as described under Methods. Additions to the standard assay mix were as indicated. All assays were performed in duplicate with 10 and 20 µg of pancreatic microsomal protein.

Figure 5. The Effect of $MnCl_2$ Concentration on the Incorporation of [^{14}C]Galactose into Desialized and Degalactosylated Fetuin

The $MnCl_2$ concentrations were varied over the range indicated. Assays were performed in duplicate with 10 and 20 μg of microsomal protein per assay. Other assay procedures were as described in Methods.


nmoles/mg protein/hour
20
15
10
5
0
10
20
30
40
MnCl2 mM

detergents tested (deoxycholate, Tween 20, Tween 60, and Tween 80) in place of Triton X-100 in the reaction mix were found to give reduced levels of enzyme activity. Dithiothreitol or mercaptoethanol were neither stimulators nor inhibitors of transferase activity.

Effect of Enzyme Concentration, Incubation Time, and pH

The galactosyltransferase has a broad pH optima as shown in Figure 6. Activity was higher with MES (Sodium 2-(N-morpholino)ethane sulfate) buffer than with Tris-HCL buffer and MES was used in all subsequent assays.

The time course for [Carbon-14]galactose incorporation into acid-precipitable and chloroform-methanol soluble material is shown in Figure 7. The low constant incorporation into the chloroform-methanol soluble fraction implies that the transfer of [Carbon-14]galactose is direct from UDP-Gal and does not involve a lipid intermediate. This possibility was further investigated by adding exogenous dolichol monophosphate to the reaction mix. After this addition no increase of radioactivity into chloroform-methanol material could be detected. Endogenous incorporation (reaction mix without DSG-fetuin) remained low at all time points.

The range of microsomal protein at which the standard galactosyltransferase assay gave linear results is illustrated in Figure 8. All galactosyltransferase assays were subsequently run in this linear protein range.

Figure 6. The Effect of pH on the Incorporation of [^{14}C]Galactose into Desialized and Degalactosylated Fetuin

Assays were performed in duplicate with 10 and 20 µg of pancreatic microsomal protein per assay using standard conditions as described in Methods. Buffers used were 0.1 M sodium 2-(N-morpholino) ethane sulfate and 0.1 M Tris-HCl.

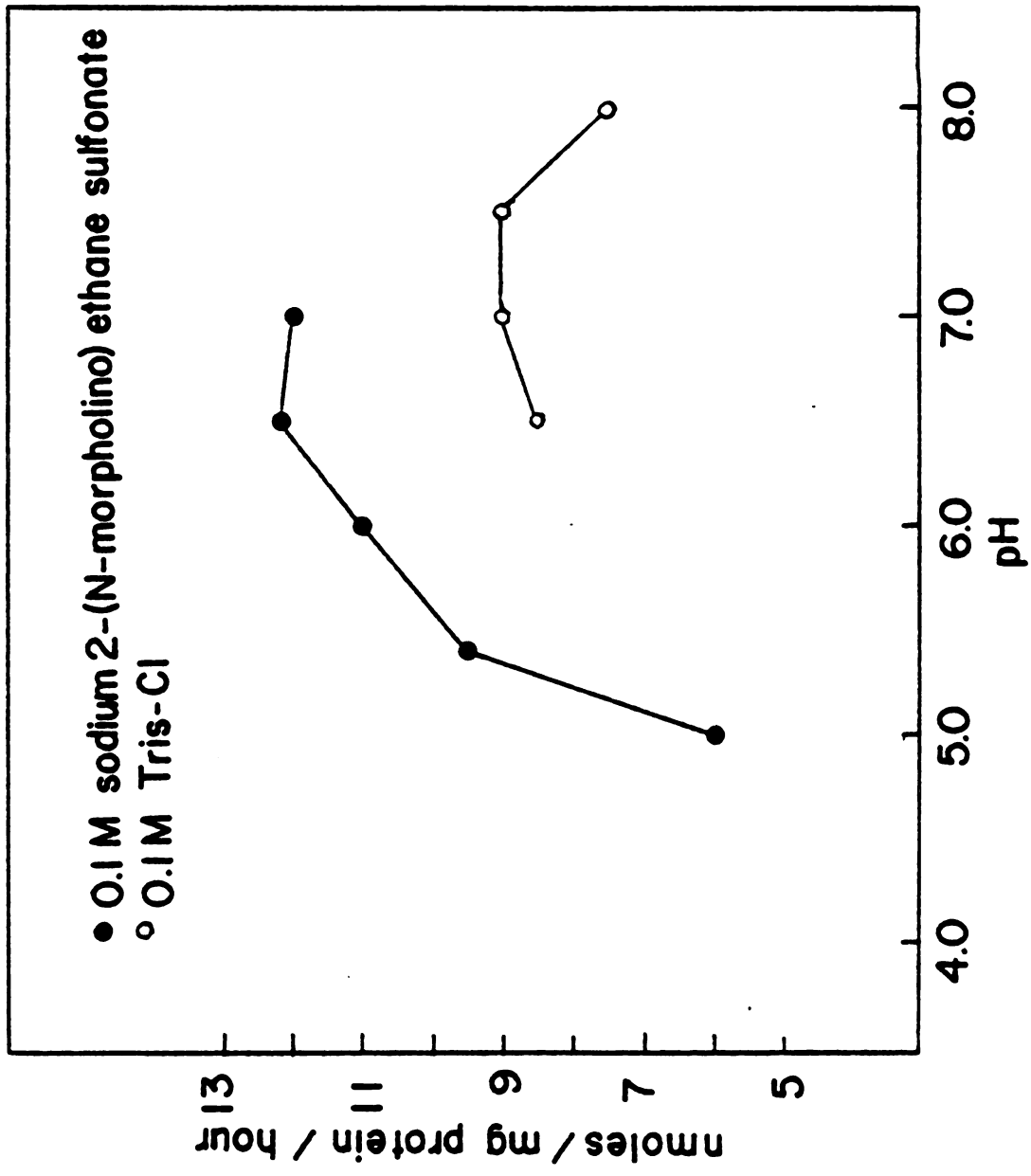

● 0.1 M sodium 2-(N-morpholino) ethane sulfonate
○ 0.1 M Tris-Cl
nmoles / mg protein / hour
5
7
9
11
13
4.0
5.0
6.0
7.0
8.0
pH

Figure 7. Time Course for Incorporation of [^{14}C]Galactose into Acid Insoluble and Chloroform-Methanol (2:1) Soluble Material

Standard galactosyltransferase assays were stopped at the time points indicated and radioactive incorporation into acid-insoluble material was determined by acid precipitation. Incorporation into the lipid phase was measured by extracting the reaction mixture with chloroform-methanol (2:1) and measuring radioactive incorporation into the lower phase. Endogenous incorporation is radioactive incorporation into acid-insoluble material in a reaction mixture without DSG-fetuin. All assays were done in duplicate with 10 μg of pancreatic microsomal protein.

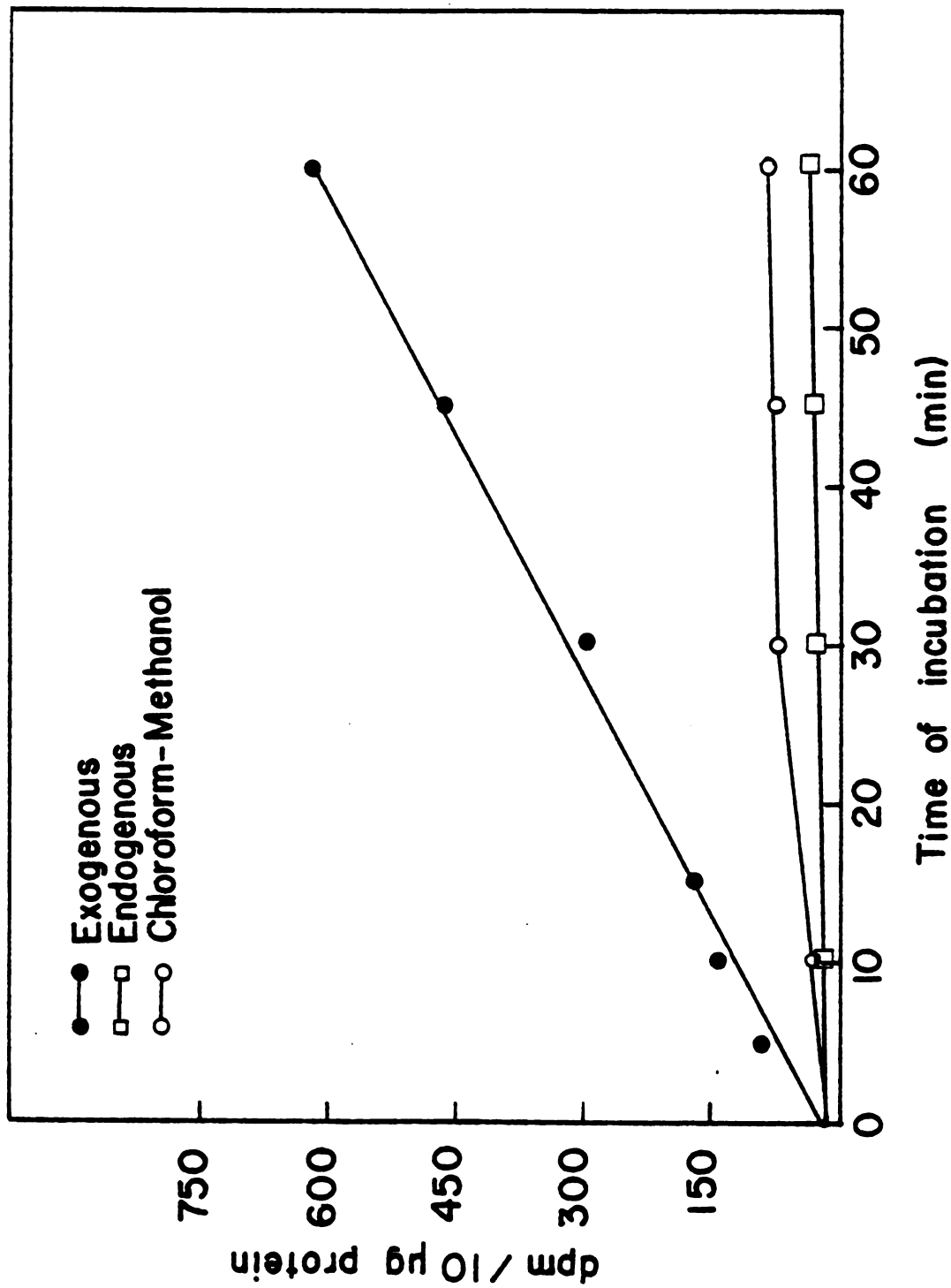
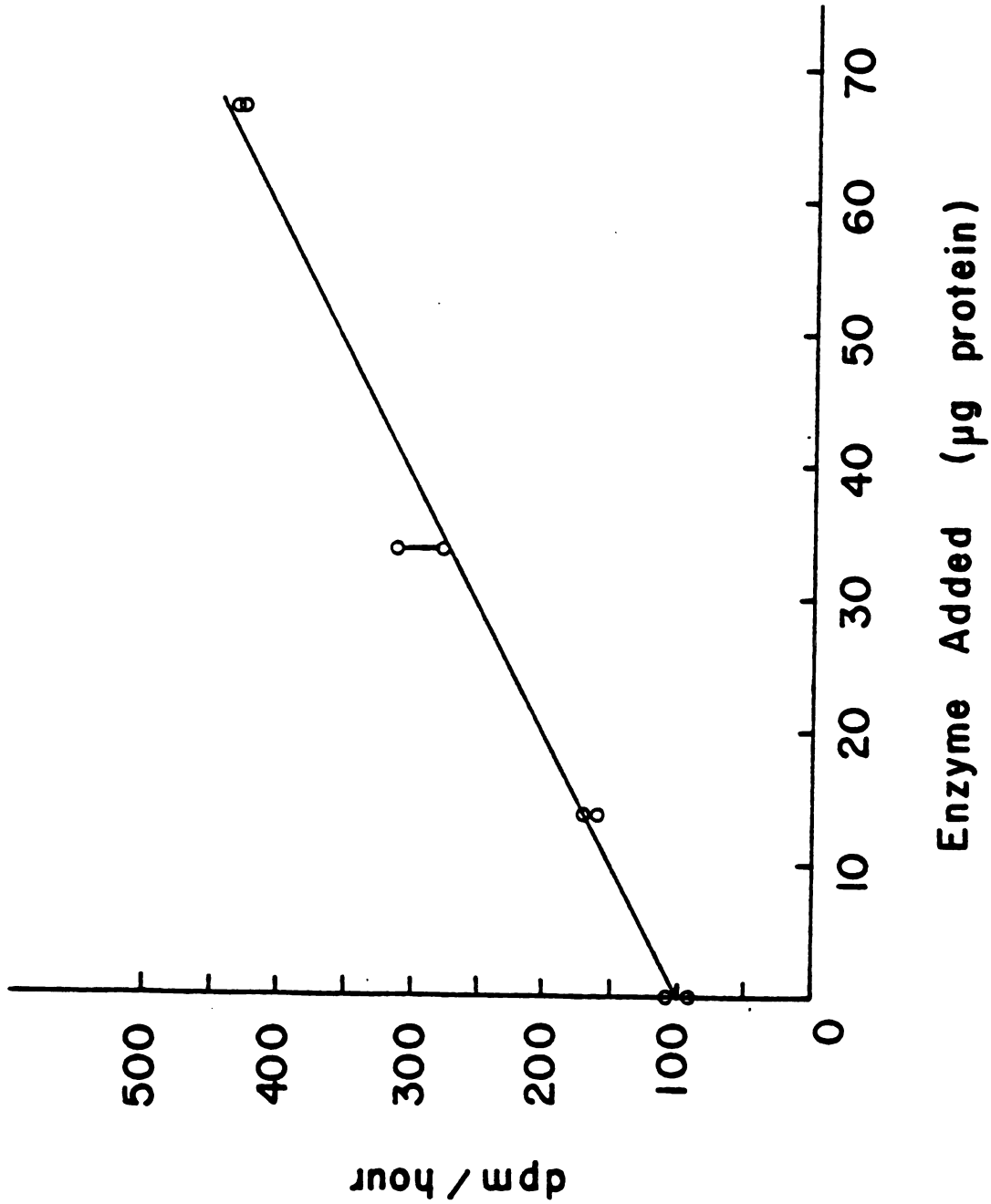

Exogenous
Endogenous
Chloroform-Methanol
dpm / 10 µg protein
150
300
450
600
750
0
10
20
30
40
50
60
Time of incubation (min)

Figure 8. Linearity of Galactosyltransferase Assay with Respect to Enzyme Concentration

Standard galactosyltransferase assays were performed as described in Methods. Enzyme source was rat pancreatic microsomes. Duplicate assays are shown.


dpm / hour
500
400
300
200
100
0
10
20
30
40
50
60
70
Enzyme Added (µg protein)

Acceptor Specificity - The activity of the galactosyltransferase when assayed with different protein substrates is summarized in Table 2. Bovine mucin minus NANA gave the highest incorporation of [Carbon-14]galactose into acid insoluble products followed by DSG-fetuin and unmodified bovine mucin. The activity towards fetuin minus NANA was less than towards native fetuin. DSG-orosomucoid had significantly less acceptor activity than DSG-fetuin. Exogenous acceptor purity was determined on SDS-polyacrylamide gels. Native fetuin (GIBCO) ran as a major band followed by two small secondary peaks while the bovine mucin was found to be extremely heterogeneous (Figure 9). Fetuin from Sigma (Type I and II) was found to be more heterogeneous than the GIBCO fetuin (data not shown) and consequently the GIBCO fetuin has been used in all studies. The endogenous activity (minus exogenous acceptor protein) was never greater than 10 percent of the total activity with DSG-fetuin present.

Exogenous Acceptor Concentration - DSG-fetuin was run in the reaction mix at concentrations from 1 mg/ml to 10 mg/ml. Transfer of [Carbon-14]galactose displayed linear kinetics over this range when up to 34 ug of pancreatic microsomal protein was assayed (Figure 10). At higher enzyme concentrations (67 ug) the assay lost its linearity. Standard assay concentration of DSG-fetuin was set at 5 mg/ml. Concentrations of DSG-fetuin above 10 mg/ml in the assay mixture were impractical as viscosity effects limited

Table 2.

Protein Substrate Specificity of Pancreatic Microsomal Galactosyltransferase

Substrate	Exposed Terminal Sugar Residue	[^{14}C]Galactose Transferred d.p.m. /mg protein/hour
Fetuin	Sialic Acid	4000
Bovine Mucin		10000
Orosomucoid		N.D.[a]
Fetuin minus NANA	Galactose	1300
Orosomucoid minus NANA		N.D.[a]
Fetuin minus NANA minus Gal	N-Acetylglucosamine	12000
Orosomucoid minus NANA minus Gal		1600
Bovine Mucin minus NANA	N-Acetylgalactosamine	20000

[a]Not determined

Figure 9. Electrophoretic Analysis of Fetuin (GIBCO) and Bovine Submaxillary Mucin (Sigma)

Samples (100 μg) were run on 7% cylindrical gels and duplicate gels were stained for protein (Coomassie blue stain) or carbohydrate (Periodic acid-Schiff). Details are described in Methods. Optical density scans of the gels was performed with a linear transport gel scanner (Gilford Instruments Model 2410 S).

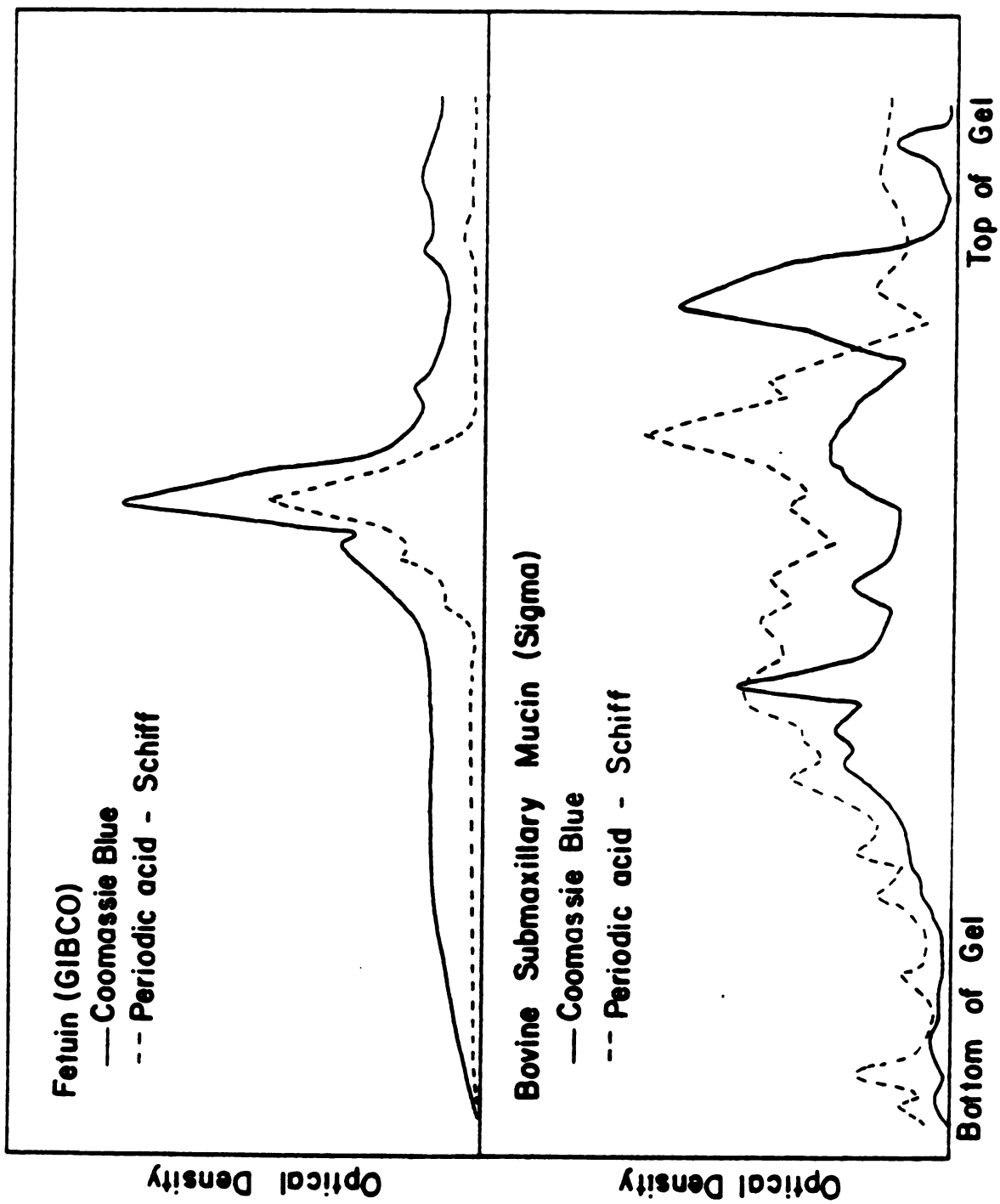
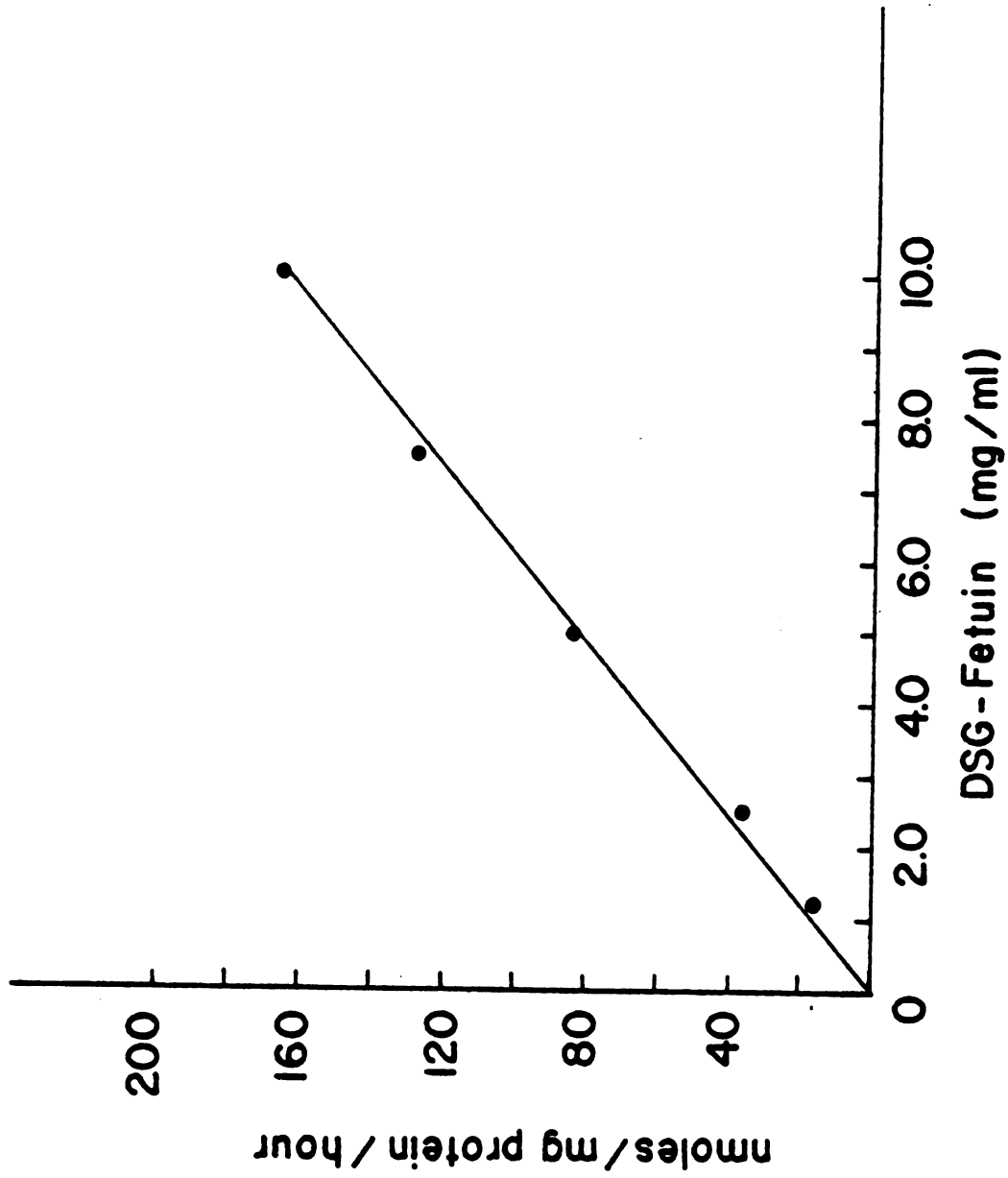

Fetuin (GIBCO)
—Coomassie Blue
-- Periodic acid - Schiff
Optical Density
Bovine Submaxillary Mucin (Sigma)
—Coomassie Blue
-- Periodic acid - Schiff
Optical Density
Bottom of Gel
Top of Gel

Figure 10. The Effect of DSG-Fetuin Concentration on Galactosyltransferase Assay Linearity

Standard galactosyltransferase assays were used as described in Methods. The concentration of DSG-Fetuin in the assay mix was as indicated.


nmoles / mg protein / hour
200
160
120
80
40
0
2.0
4.0
6.0
8.0
10.0
DSG-Fetuin (mg/ml)

the practicality of the assay.

Stability - The transferase was remarkably stable when stored as a microsomal suspension in 0.3 M sucrose. After 24 hours at 23 degrees C over 90 percent of activity was retained. After two weeks at -20 degrees C over 80 percent of activity remained. Activity remained unchanged when the microsomal suspension was stored up to one year at -80 degrees C.

Identification of Labeled Galactose Incorporated into Protein

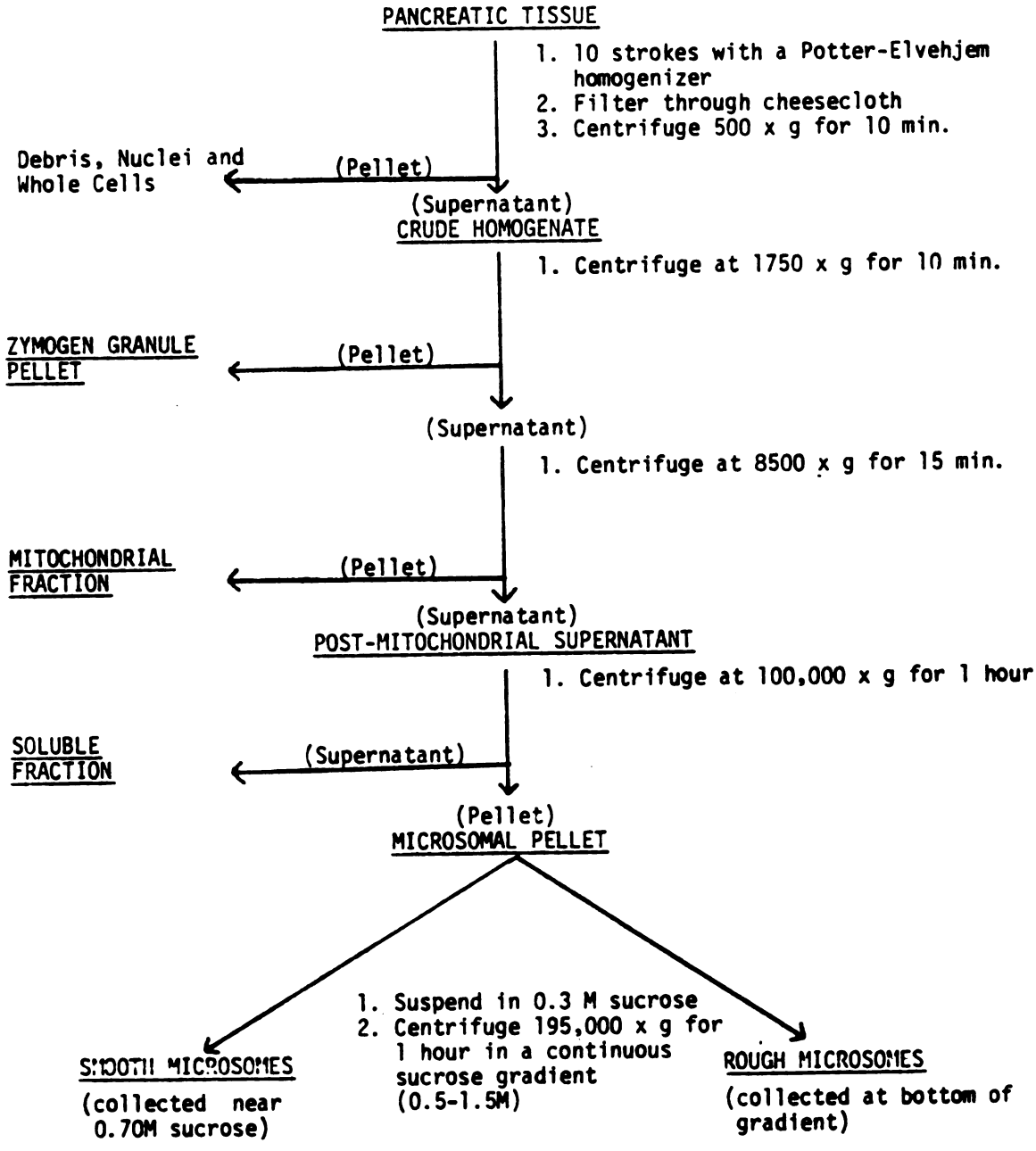
The product of the DSG-fetuin galactosyltransferase assay was analyzed to rule out the possibility that the radioactivity measured in the product was a metabolic product of the labeled UDP-galactose. Labeled DSG-fetuin was hydrolyzed in 4 M HCl at 100 degrees C for 3 hours. After hydrolysis the sample was neutralized with silver carbonate and subsequent paper chromatography revealed a single radioactive spot migrating with standard galactose (data not shown).

Subcellular Localization of Galactosyltransferase Activity

To determine the subcellular distribution of galactosyltransferase activity, rat exocrine pancreatic tissue was fractionated as outlined in Figure 11. The resulting fractions were assayed for galactosyltransferase activity

Figure 11. Sub-Cellular Fractionation of Rat Pancreatic Tissue

The fractionation scheme used was that of Jamieson and Palade (42) as modified by Ronzio (193). Details are in Methods.


PANCREATIC TISSUE
1. 10 strokes with a Potter-Elvehjem homogenizer
2. Filter through cheesecloth
3. Centrifuge 500 x g for 10 min.
Debris, Nuclei and Whole Cells
(Pellet)
(Supernatant)
CRUDE HOMOGENATE
1. Centrifuge at 1750 x g for 10 min.
ZYMOGEN GRANULE PELLET
(Pellet)
(Supernatant)
1. Centrifuge at 8500 x g for 15 min.
MITOCHONDRIAL FRACTION
(Pellet)
(Supernatant)
POST-MITOCHONDRIAL SUPERNATANT
1. Centrifuge at 100,000 x g for 1 hour
SOLUBLE FRACTION
(Supernatant)
(Pellet)
MICROSOMAL PELLET
1. Suspend in 0.3 M sucrose
2. Centrifuge 195,000 x g for 1 hour in a continuous sucrose gradient (0.5-1.5M)
SMOOTH MICROSOMES
(collected near 0.70M sucrose)
ROUGH MICROSOMES
(collected at bottom of gradient)

and the results are shown in Table 3. The debris pellet had a similar specific activity to that of the crude homogenate. This fraction was largely made up of undisrupted whole cells and connective tissue. The zymogen granule fraction contained very little activity and other studies with purified zymogen granule membranes (148) have shown similar results. Galactosyltransferase had little activity in the post-mitochondrial supernatant fraction as would be expected for a membrane-bound enzyme. Clearly, most of the recoverable activity (from the crude homogenate) was found in the crude microsomal fraction with an enrichment of specific activity by a factor of almost eleven. The rough microsomal fraction had essentially the same activity as did the crude microsomal mixture. The smooth microsomal fraction contained less than 4 percent of the total microsomal activity, yet gave a relative enrichment of 30 fold in specific activity over the crude homogenate. The smooth microsomal fraction was composed of Golgi, plasma membrane, and ribosome-free endoplasmic reticulum. Terminal galactosyltransferase activity is considered to be generally localized in the Golgi fraction, however, localization in other membrane fractions could not be discounted.

Table 3

Distribution of DSG-Fetuin: Galactosyltransferase in Pancreatic Subcellular Fractions.[a]

Fraction	Total Activity[b]	% of Total Activity[c]	Specific Activity[d]	Relative Activity[e]
Crude Homogenate	2000	100	2.9	1.0
Debris Pellet	280	14	3.4	1.2
Zymogen Granule Fraction	10	0.1	0.5	0.2
Post Mitochrondrial Supernatant	25	1	0.3	0.1
Crude Microsomes	800	41	10.8	3.7
Rough Microsomal Fraction	215	10	9.6	3.3
Smooth Microsomal Fraction	35	2	87.0	29.7

[a]Fractionation procedure is described in Methods. Galactosyltransferase assays were run under standard conditions as described in Methods.

[b]nmoles galactose transferred per hour

[c]Activity of fraction/Activity of Crude Homogenate

[d]nmoles galactose transferred per mg protein per hour

[e]Specific Activity of Fraction/Specific Activity of Crude Homogenate

Molecular Exclusion Chromatography of Solubilized Galactosyltransferase

Multiple forms of galactosyltransferase have been detected in other systems where the enzyme exists in a soluble form (161). The possibility that multiple forms of the microsomal galactosyltransferase might also exist was explored by running solubilized microsomes over a Biogel P-150 molecular exclusion column. A broad peak of activity was observed immediately after the void volume (data not shown).

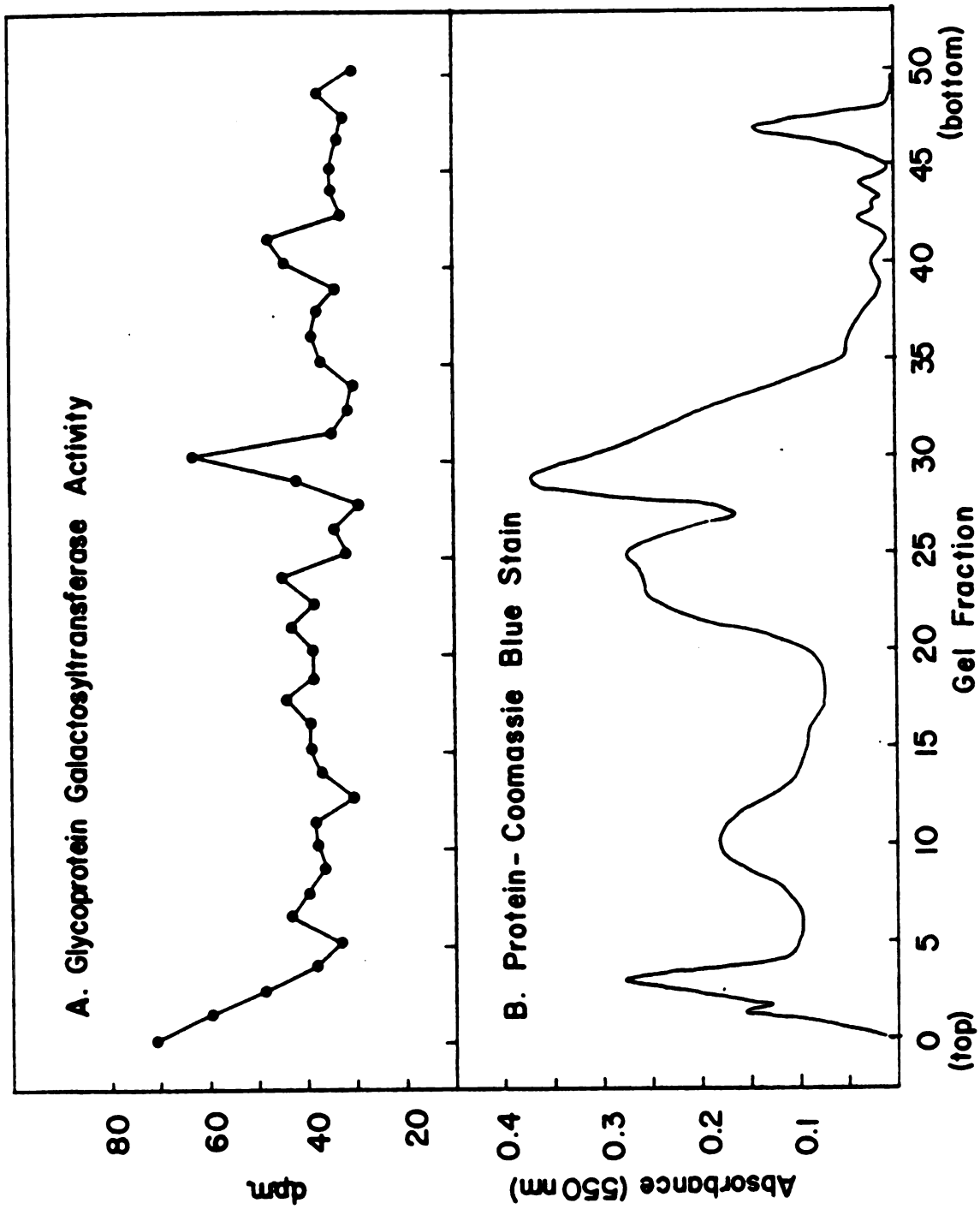
Polyacrylamide Gels of Solubilized Galactosyltransferase

Solubilized microsomes were analyzed on 7 percent polyacrylamide gels and galactosyltransferase activity localized by slicing the gels into fractions and incubating the minced fractions with a galactosyltransferase reaction mixture. Multiple forms of serum galactosyltransferase have been distinguished on polyacrylamide gel by other investigators (162). The results of a typical gel are shown in Figure 12. One or perhaps two bands of transferase activity were seen. The recovery of activity from the gels was extremely low (less than 10 percent) under all conditions examined.

Figure 12. Recovery of Galactosyltransferase Activity from Polyacrylamide Gels

Rat pancreatic microsomes were solubilized with 5% Triton X-100 and samples (100 μg) run on 7% polyacrylamide gels. Gels were sliced into fractions and incubated with the galactosyltransferase reaction mixture. Incorporation into acid insoluble material is shown in panel A.

Duplicate gels were stained with Coomassie blue protein stain and scanned with a linear transport (Gilford Instruments, model 2410 s).-panel B


A. Glycoprotein Galactosyltransferase Activity
dpm
80
60
40
20
B. Protein-Coomassie Blue Stain
Absorbance (550 nm)
0.4
0.3
0.2
0.1
0 (top)
5
10
15
20
25
30
35
40
45
50 (bottom)
Gel Fraction

Isoelectric Focusing of Solubilized Galactosyltransferase

Solubilized enzyme was run on an isoelectric focusing column to determine an isoelectric point for the enzyme as well as if multiple forms might be separated by charge. Figure 13 demonstrates that a broad though well defined band of activity ran between pH 6.0 and pH 8.0 with a maximum galactosyltransferase activity at pH 6.9 to 7.0.

Pyrophosphate Activity in Microsomal Preparations

Since the presence of pyrophosphatase activity would hydrolyze UDP-galactose, the extent of pyrophosphatase activity was measured in the microsomal preparations. AMP and ATP were examined for their inhibition of pyrophosphatase activity and their effect on galactosyltransferase activity. Standard protocol for the galactosyltransferase assay was used except that inhibitors were present in the concentrations indicated. The assays were stopped by the addition of ethanol-acetic acid (5 percent) rather than TCA-PTA in the galactosyltransferase assay. This alteration was found to be necessary as the TCA rapidly hydrolyzed the UDP-galactose upon addition to the reaction mix. Immediately after quenching the assay, the entire reaction mix was run on a paper chromatographic system which clearly separated UDP-galactose, galactose-1-phosphate, and galactose. Control scans of three paper chromatograms are shown in Figure 14. The rate of hydrolysis of UDP-galactose

Figure 13. Isoelectric Focusing of Solubilized Galactosyltransferase

Rat pancreatic microsomes solubilized in 5% Triton X-100 were run on a sucrose density gradient isoelectric focusing column. Fractions were collected from the column, assayed in the standard galactosyltransferase assay mix, and incorporation into acid insoluble material determined for each fraction. Details are found in Methods.

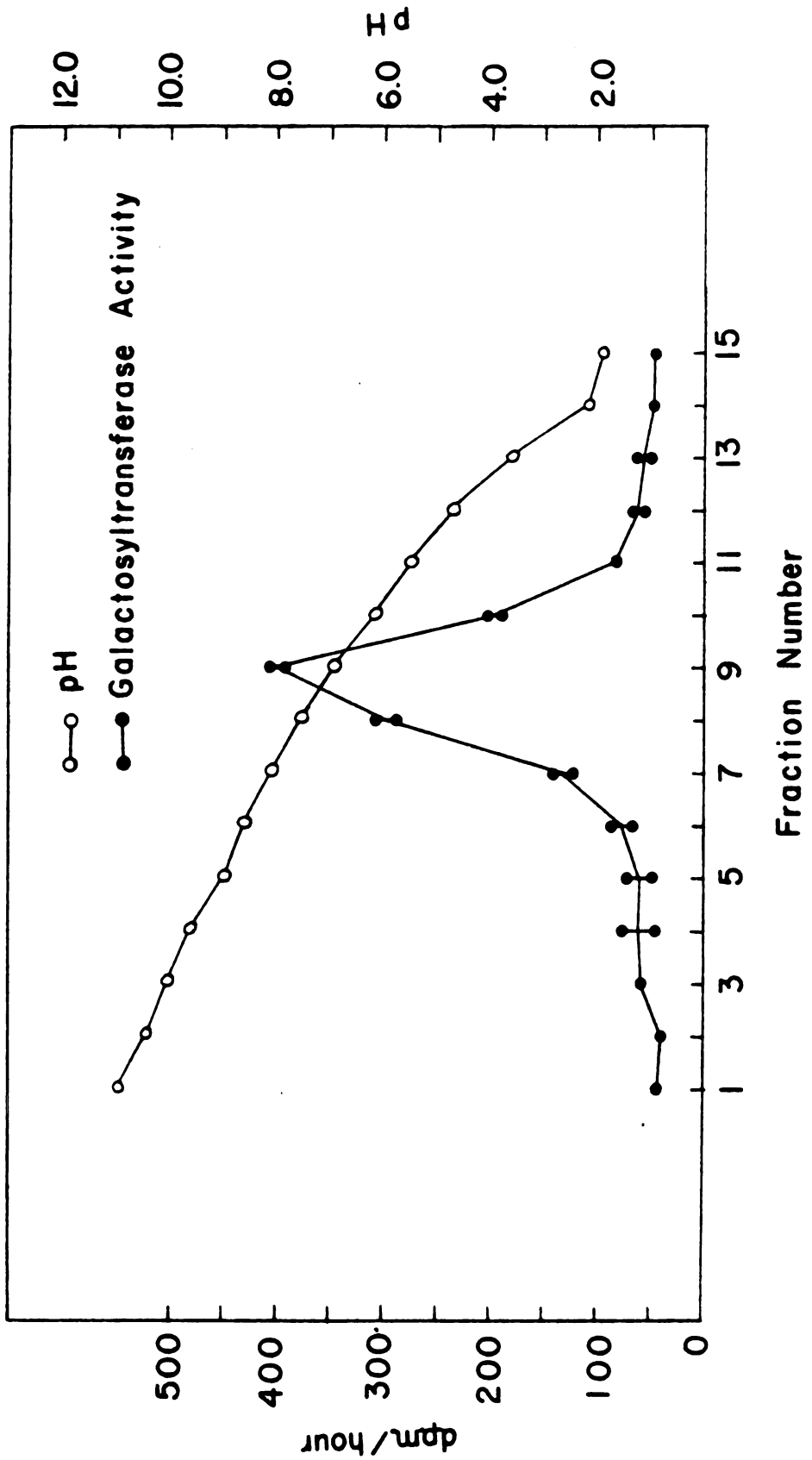
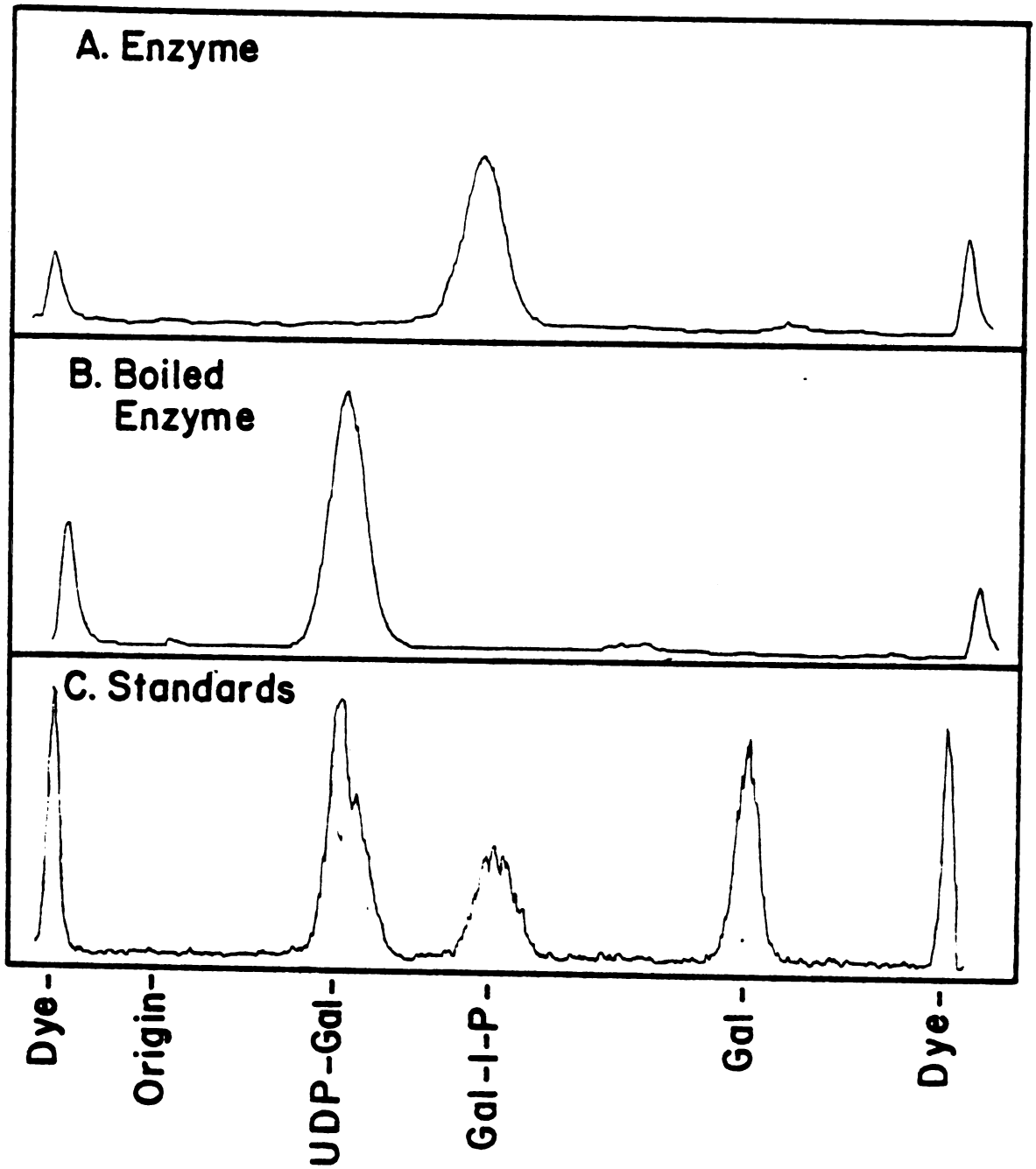

pH
Galactosyltransferase Activity
dpm / hour
0
100
200
300
400
500
pH
2.0
4.0
6.0
8.0
10.0
12.0
1
3
5
7
9
11
13
15
Fraction Number

Figure 14. Radiochromatographic Scans for the UDP-galactose Pyrophosphatase Assay

Incubation and assay conditions are described in Methods.
Panel A- 10 μg pancreatic microsomal protein with UDP-[^{14}C] galactose (22,000 dpm)
Panel B- Conditions are the same as for panel A except the microsomal protein was heated in a boiling water bath for 3 minutes before addition to the assay mix.
Panel C- Radioactive standards- All New England Nuclear


A. Enzyme
B. Boiled Enzyme
C. Standards
Dye-
Origin-
UDP-Gal-
Gal-1-P-
Gal-
Dye-

is shown to be proportional to incubation time in Figure 15.

The effect of 3.3mM adenosine triphosphate (ATP) and 3.4 MM adenosine (AMP) on galactosyltransferase activity is summarized in Table 4. Radiochromatographic scans of the pyrophosphatase assay in the presence of AMP and unlabeled UDG-Gal is shown in Figure 16. The course of hydrolysis of UDP-galactose in the presence of 3.3 mM ATP and 3.4 mM AMP is shown in Figure 17. After scanning with a radiochromatogram scanner (Packard Model 7201), the paper strips were cut into sections and counted by liquid scintillation spectrophotometry. All assays were done in duplicate. The results indicate that neither AMP nor ATP at the concentrations tested inhibited or stimulated galactosyltransferase activity in the absence of appreciable pyrophosphatase activity. In most cases the large excess of unlabeled UDP-galactose (4 mM) was sufficient to prevent excess hydrolysis of labeled substrate (UDP-[Carbon-14]galactose) by pyrophosphatase activity. AMP was determined to be a better inhibitor of pyrophosphatase activity than ATP and was used in subsequent galactosyltransferase assays when pyrophosphatase activity was high. A summary of the pyrophosphatase data is presented in Table 5.

Figure 15. Radiochromatographic Scans of the UDP-galactose Pyrophosphatase Assay - Time Course for Enzymatic Hydrolysis of UDP-Gal

All assays contained 10 µg of pancreatic microsomal protein plus 4.0 mM UDP-Gal.
Panel A - No incubation
Panel B - 15 minute incubation(37 degrees C)
Panel C - 30 minute incubation(37 degrees C)

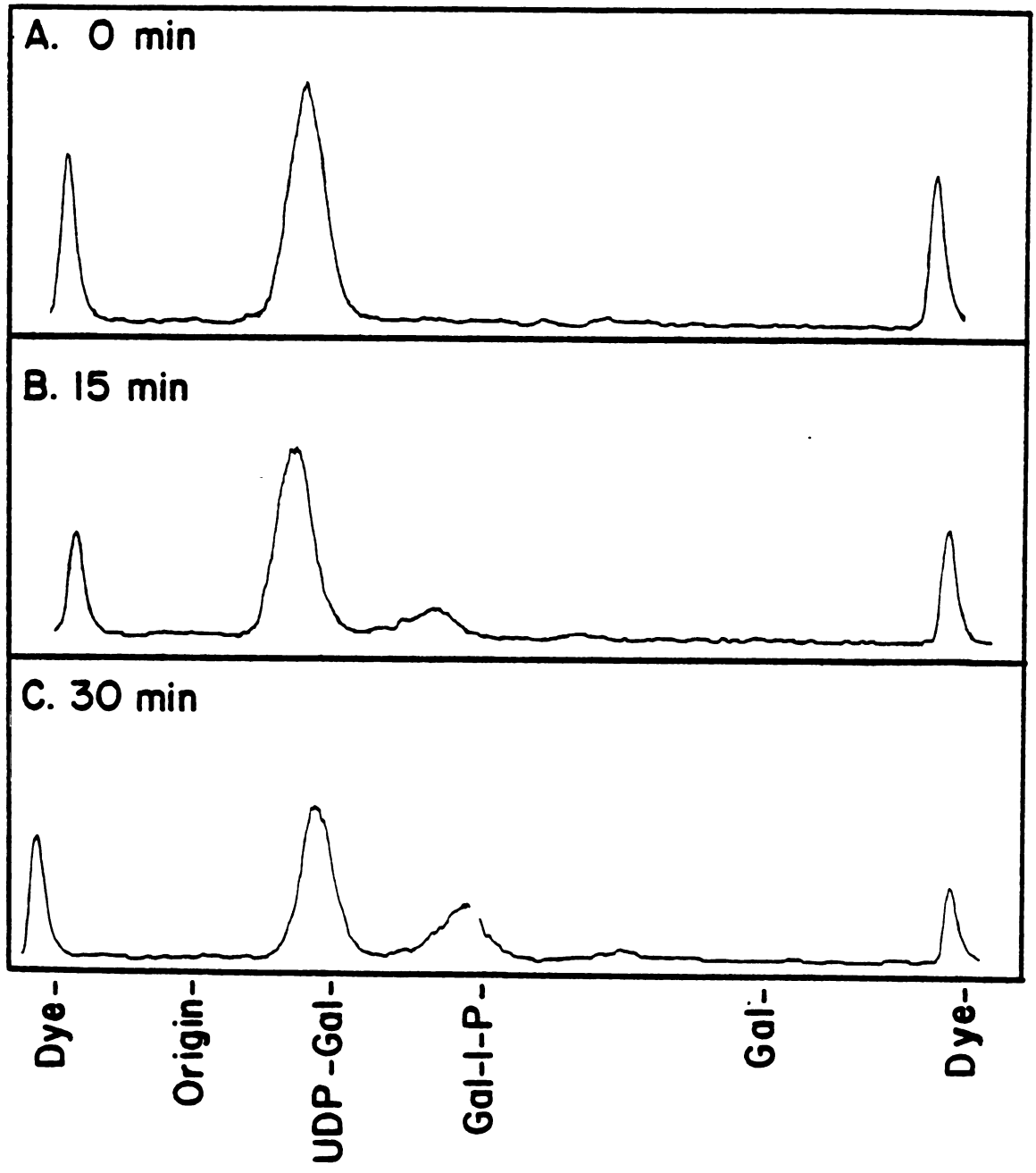

A. 0 min
B. 15 min
C. 30 min
Dye-
Origin-
UDP-Gal-
Gal-1-P-
Gal-
Dye-

Table 4

Effect of AMP and ATP on Pyrophosphatase Activity

Assay	% UDP-Gal Remaining After:			% UDP-Gal Hydrolyzed
	0 min	15 min	30 min	30 min
Boiled Enzyme	95.0	94.8	93.9	1.1
Enzyme	93.7	77.8	62.7	31
Enzyme + ATP(4.0mM)	96.7	89.5	82.8	13.9
Enzyme + AMP(4.0mM)	95.8	94.1	92.2	3.6

Table 5

Effect of AMP and ATP on Galactosyltransferase Activity

Assay	[^{14}C]Galactose Transferred (d.p.m.) per:		
	25 µg Protein	40 µg Protein	60 µg protein
Reaction Mix	1540	2025	2400
Reaction Mix + AMP(4.0mM)	1550	2000	2425
Reaction Mix + ATP(4.0mM)	1300	1820	2425

Figure 16. Radiochromatographic Scans for the UDP-galactose Pyrophosphatase Assay - Effect of AMP and Excess Unlabeled UDP-Gal on Pyrophosphatase Activity

Panel A - Standard reaction mix plus 3.4mM AMP
Panel B - Standard reaction mix plus 4.0 mM UDP-Gal(unlabeled)
Panel C - Standard reaction mix
All assays contained 10 μg of pancreatic microsomal protein.

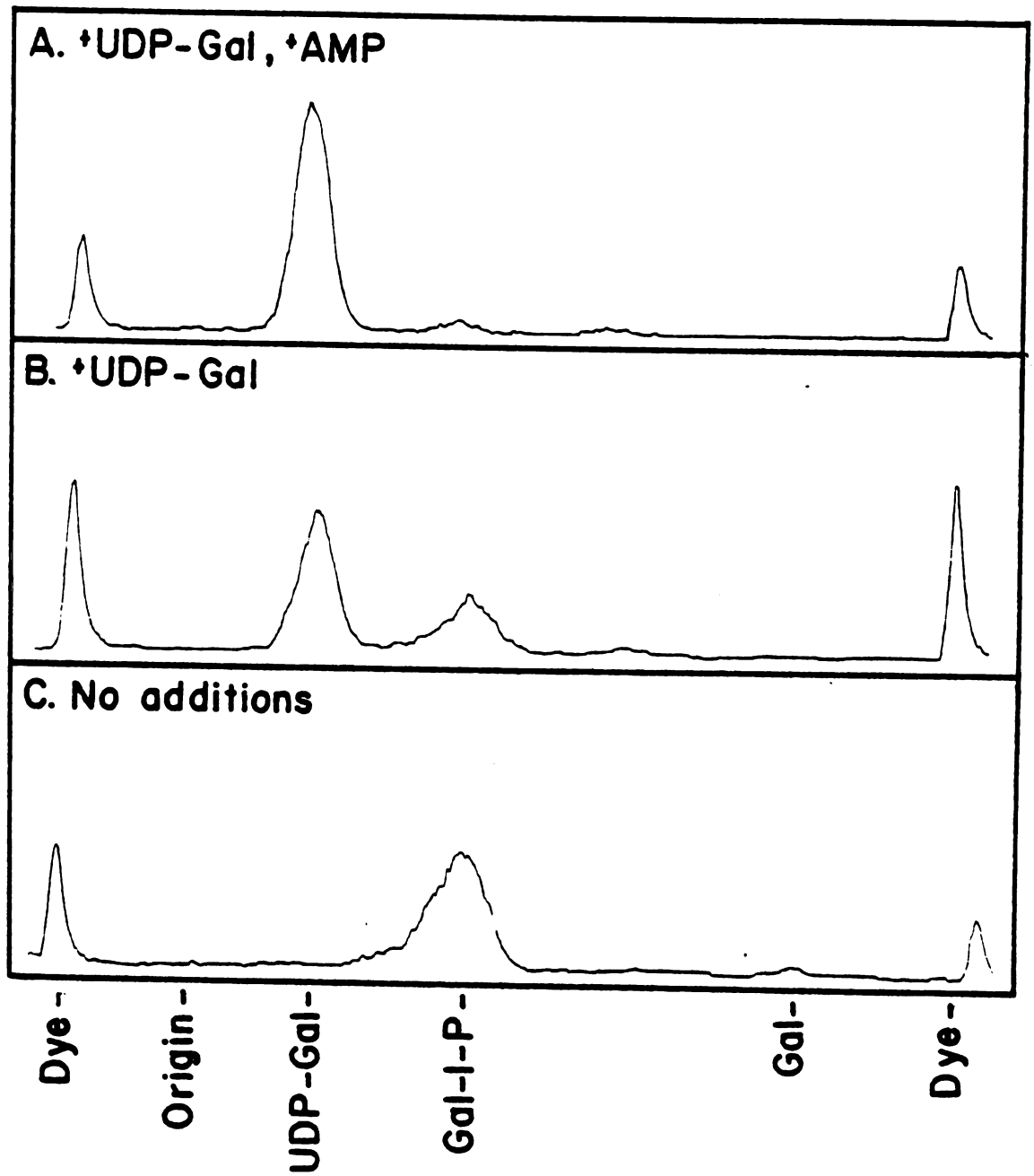
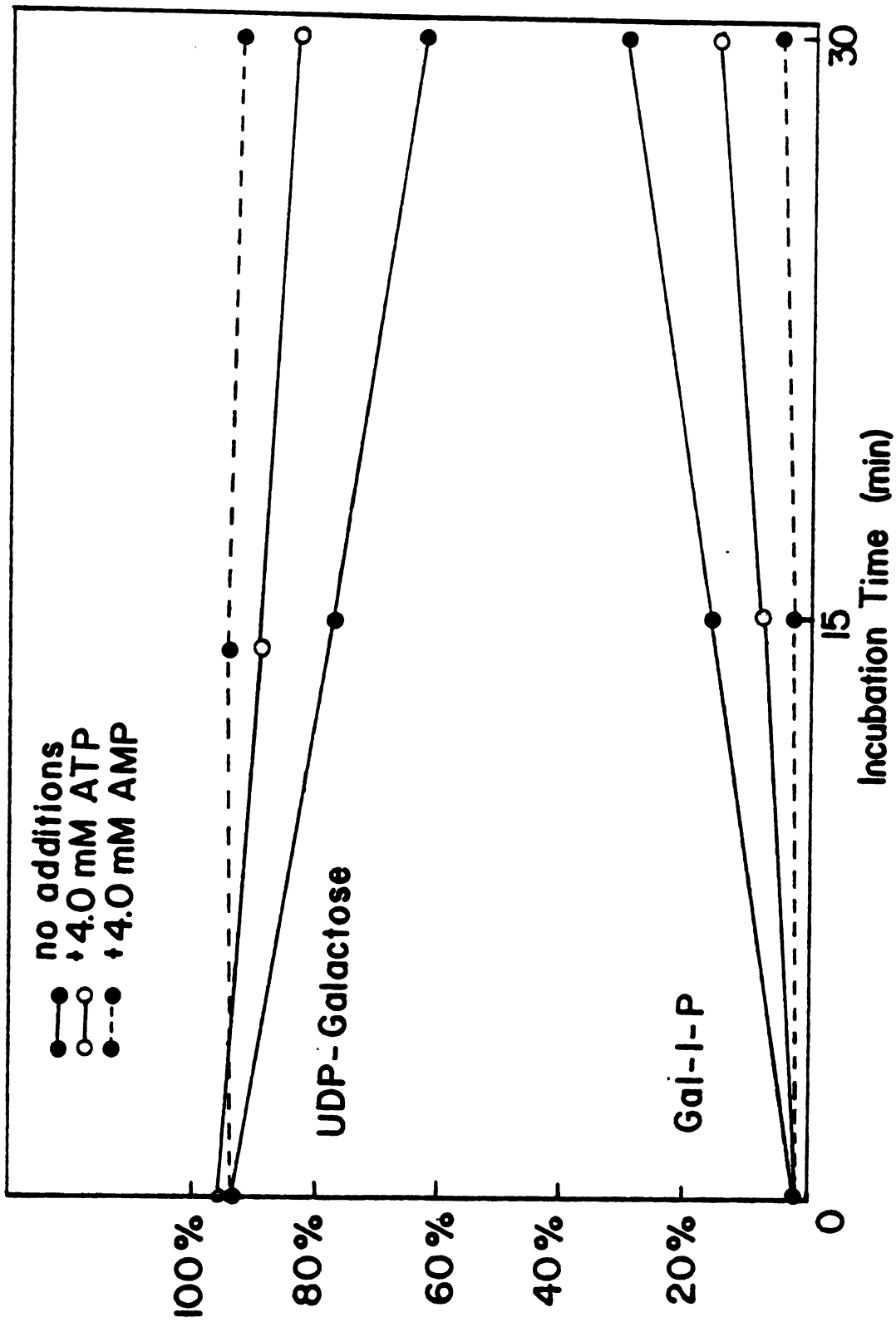

A. +UDP-Gal, +AMP
B. +UDP-Gal
C. No additions
Dye-
Origin-
UDP-Gal-
Gal-1-P-
Gal-
Dye-

Figure 17. Inhibition of Pyrophosphatase Hydrolysis of UDP-Galactose by AMP and ATP

Standard reaction mixtures (± ATP or AMP) were utilized without unlabeled UDP- Gal. Assays received 10 ug of pancreatic microsomal protein and were terminated at 0, 15, and 30 minutes and run on descending paper chromatograms. After development the areas corresponding to UDP-Gal or Gal-1-P were cut out and radioactivity quantitated by liquid scintillation spectrometry. Details of procedures are in Methods. No appreciable degradation of Gal-1-P occurred after 30 minutes.


no additions
+4.0 mM ATP
+4.0 mM AMP
UDP-Galactose
Gal-1-P
100%
80%
60%
40%
20%
0
15
30
Incubation Time (min)

Galactosyltransferase Levels in Rat Embryo Pancreatic Homogenates

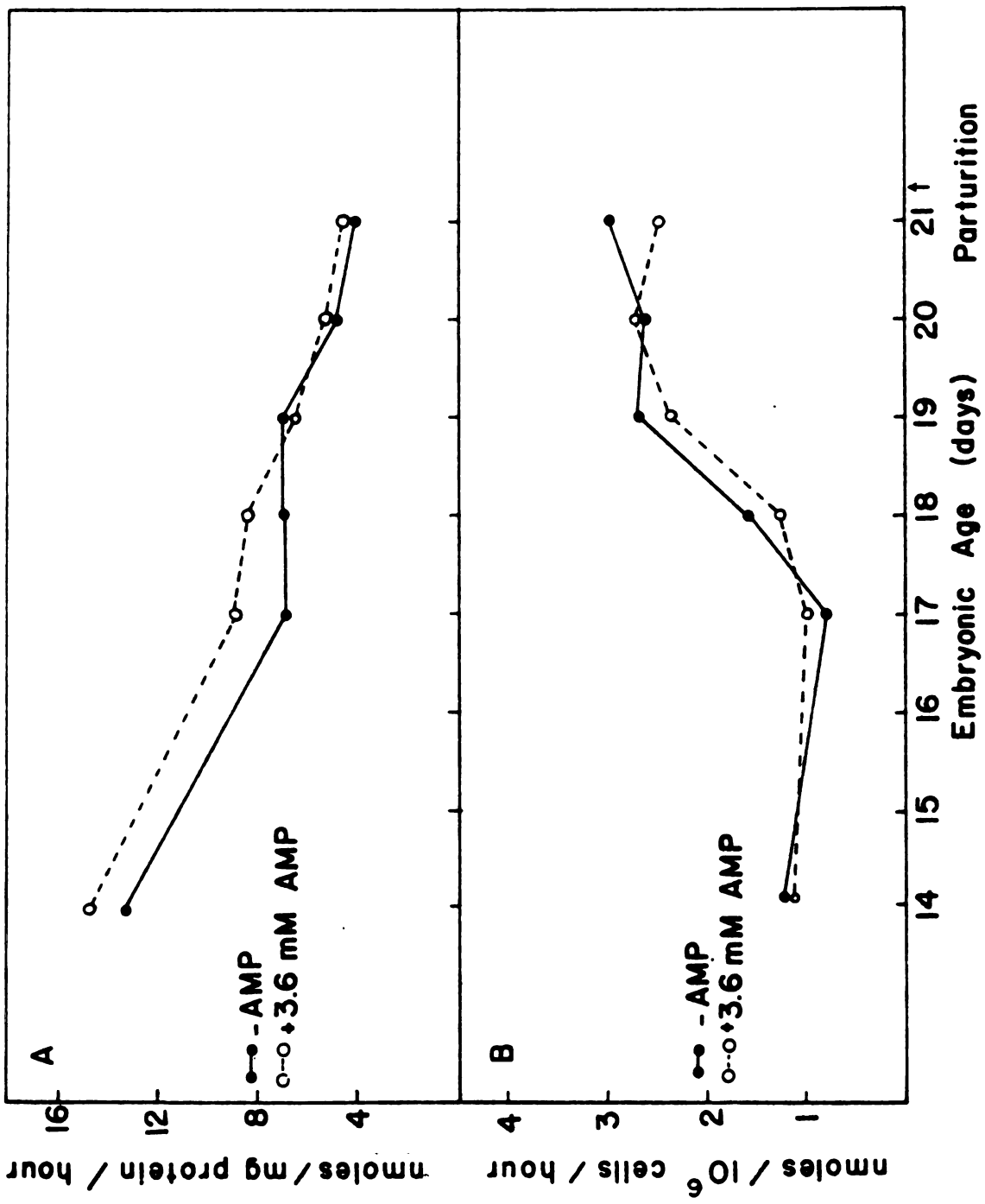
Rat embryos were dissected from Sprague-Dawley females whose pregnancies had been timed from fertilization. The small size of the tissues made microsomal preparations impractical at early embryonic periods and consequently whole pancreatic homogenates were used in the assays. Pancreatic tissue from 5 to 30 embryos (depending on embryonic age) was pooled, disrupted by sonication and assayed for enzymatic activity.

Specific activity of DSG-fetuin galactosyltransferase as a function of embryonic age is given in Figure 18-A. The decline in specific activity from day 14 to birth was linear from 14 nmoles/mg protein/hour (day 14) to 5 nmoles/mg protein/hour (day 21, parturition at day 21 to day 22). This decline was identical in the presence or absence of the pyrophosphatase inhibitor AMP.

Tissue differentiation during development can occur with concommitent cell division and protein synthesis or by cell division with little "de novo" protein synthesis. Since the latter is known to occur in pancreatic development the specific activity of galactosyltransferase was calculated on the basis of cell number rather than total protein. Activity of galactosyltransferase as a function of embryonic age increased over the development period as shown in Figure 18-B. This duplicates the basic pattern first observed by Carlson (98).

Figure 18. Galactosyltransferase Activity in the Embryonic Rat Pancreas

Galactosyltransferase activities were measured in embryonic rat pancreas homogenates (± 3.6 mM AMP) as a function of embryonic age. Panel A shows activity calculated as a function of mg protein and panel B shows activity as a function of cell number.


A
B
-AMP
+3.6 mM AMP
nmoles / mg protein / hour
nmoles / 10^6 cells / hour
16
12
8
4
4
3
2
1
14
15
16
17
18
19
20
21
Embryonic Age (days)
Parturition

Galactosyltransferase Levels in Rat Neonate Pancreatic Homogenates

Pancreases were dissected from rat pups with neonate day 1 being defined as day of parturition. Pancreases were homogenized using a Potter-Elvehem homogenizer or were disrupted by sonication as with the embryonic samples. Assays yielded identical results regardless of type of disruption employed.

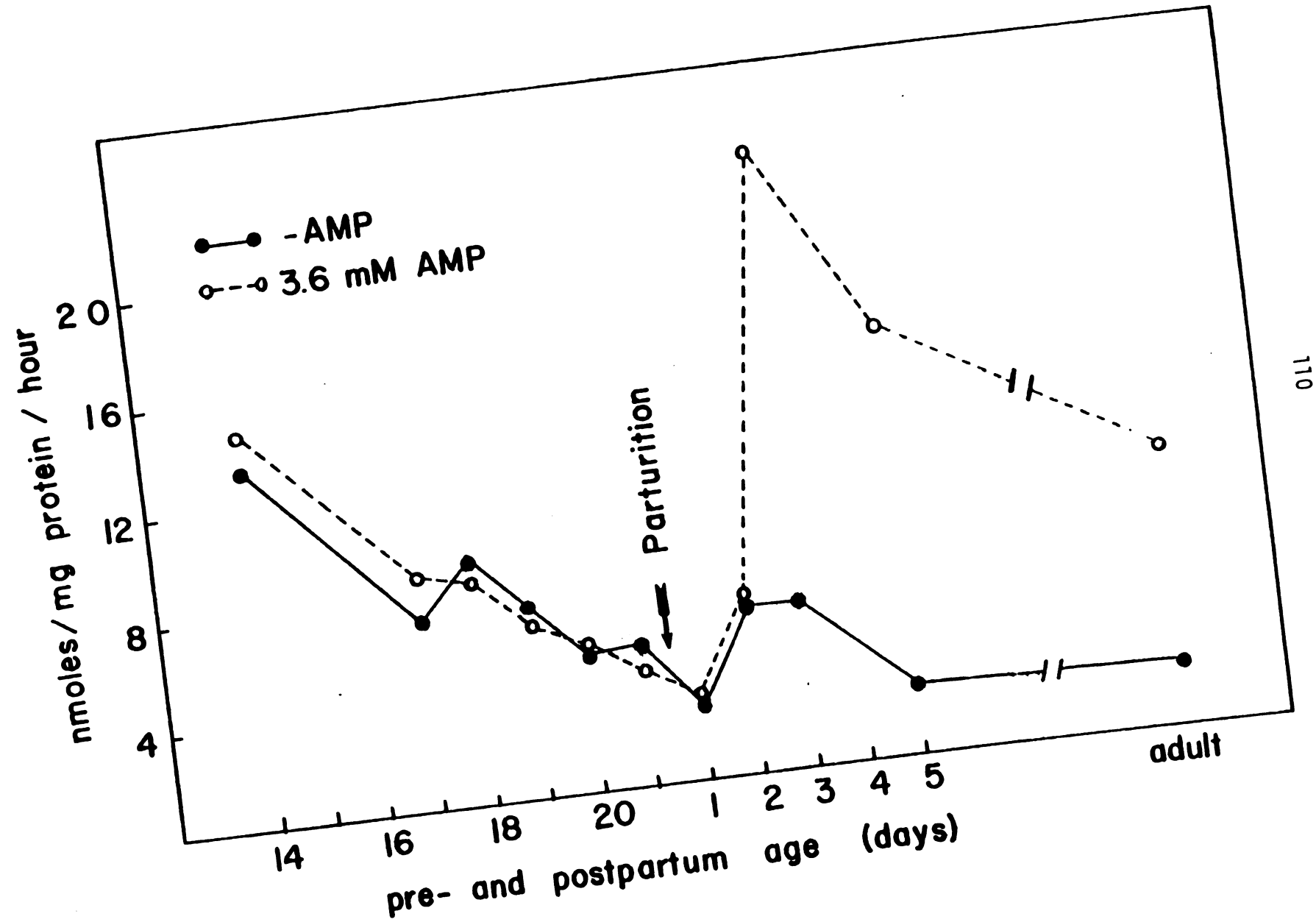
The activity of galactosyltransferase in the absence of inhibitors of pyrophosphatase (AMP) remained fairly constant throughout early neonatal age. However, when 3.3 mM AMP was included in the reaction mix, an increase in galactosyltransferase activity was noted (Figure 19). This rise in activity reached a maximum on neonate day 3 or 4 and subsequently declined to adult levels by day 7 or 8.

DSG-orosomucoid as a glycoprotein acceptor was also assayed with the neonates. The same rise was observed at day 3 or 4 but of a lesser magnitude than with DSG-fetuin. These data are compatible with the differences in enzyme acceptor specifics as noted for adult tissue in Table 2. This observation suggests that the acceptor specificity of the galactosyltransferase is apparently unchanged during the neonatal rise in enzymatic activity.

In the developing chicken brain a developmental transition occurs in which glycosyltransferases change from membrane-bound to a soluble species (103). To test this hypothesis in developing rat pancreas, homogenates from late

Figure 19. Glycoprotein Galactosyltransferase Activity in the Embryonic and Neonatal Rat Pancreas

Pancreas were removed from embryonic and neonatal rats, homogenized and assayed for glycoprotein galactosyltransferase activity as described in Methods. Duplicate samples were run, one in the presence of 3.6 mM AMP. The difference in galactosyltransferase levels between assays run ± AMP are due to hydrolysis of the UDP-Gal substrate by pyrophosphatase activity.


-AMP
3.6 mM AMP
nmoles / mg protein / hour
20
16
12
8
4
Parturition
14
16
18
20
1
2
3
4
5
adult
pre- and postpartum age (days)

embryo (20 day) through neonates (day 6) were separated into soluble and membrane fractions. As shown in Figure 20, the activity was concentrated in the microsomal fraction with little activity being found in the microsomal supernatant. Consequently no evidence was found for a similar transition to a soluble form of the enzyme in the neonates.

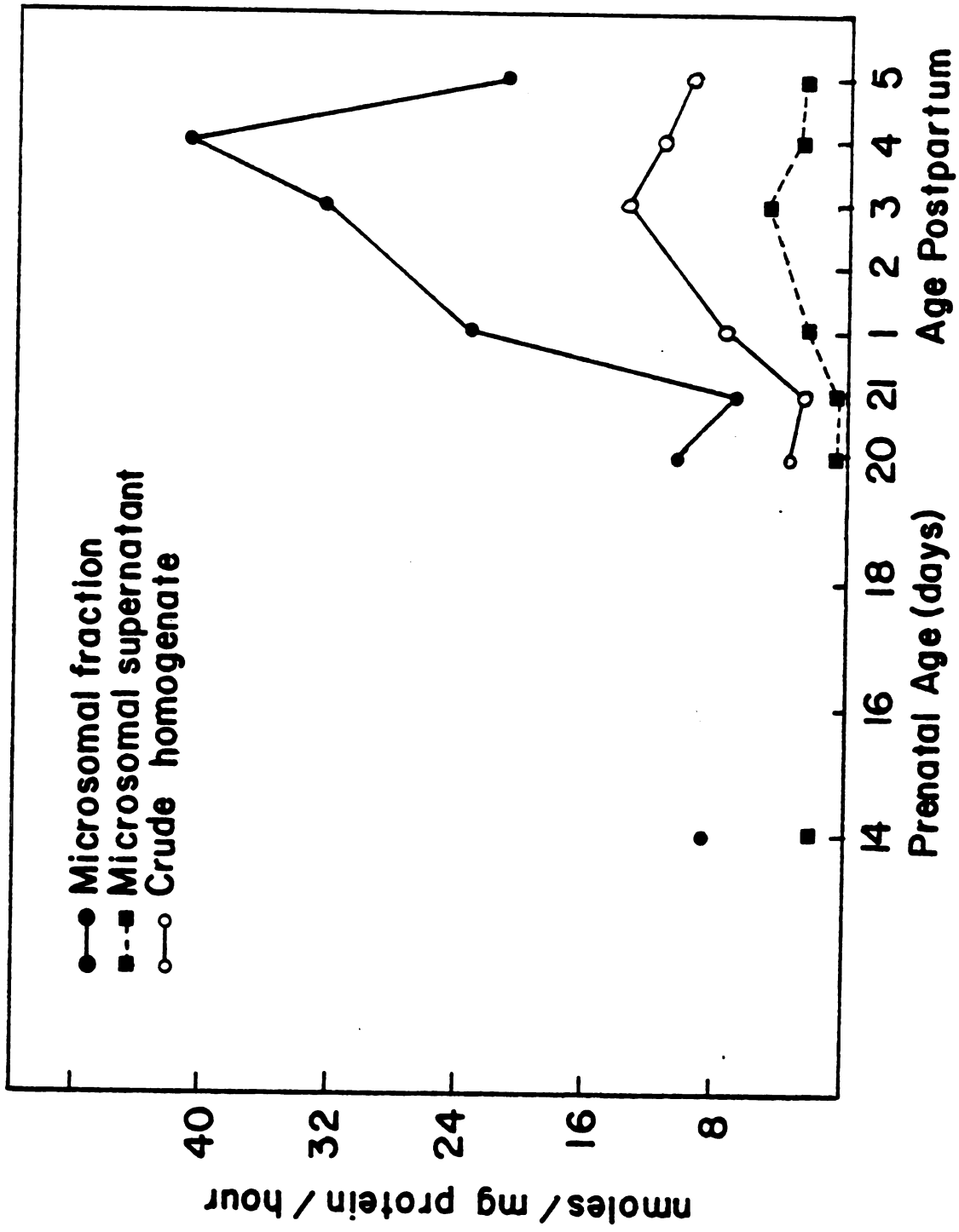
The possibility was then explored that an activator of galactosyltransferase was being synthesized during the neonatal rise in enzymatic activity. A series of mixing experiments were performed to determine if a soluble activator or inhibitor of galactosyltransferase was present during late embryonic or early neonatal periods. No evidence was found for the presence of an activator or inhibitor of galactosyltransferase activity in the neonate day 3 pancreas.

The possibility that a new distinct galactosyltransferase was being synthesized in the neonates and that the elevated levels were the result of an additive activity between the embryonic and the adult form was explored by characterizing the neonatal activities by SDS-polyacrylamide gel electrophoresis and isoelectric focusing. Results supported one enzyme form with characteristics identical to those reported earlier for the adult form.

Pyrophosphatase Activity in the Neonate Pancreas - Since the rise in galactosyltransferase activity at day 3 or day 4 was concommitant with a rise in apparent pyrophosphatase activity (indicated by the difference in activity between

Figure 20. Glycoprotein Galactosyltransferase Activity in Sub-Cellular Fractions of the Late Embryonic and Neonatal Rat Pancreas

In order to determine if transitions occur between soluble and membrane bound forms of galactosyltransferase activity, late embryonic and neonatal rat pancreas samples were homogenized, and after low speed centrifugation (5,000 x g) to remove debris, were seperated into the microsomal fraction (100,000 x g pellet) and the microsomal supernatant fraction (100,00 x g supernatant). Galactosyltransferase assays were run on these fractions (plus 4.0 mM AMP) as described in Methods.


Microsomal fraction
Microsomal supernatant
Crude homogenate
nmoles / mg protein / hour
40
32
24
16
8
14
16
18
20
21
1
2
3
4
5
Prenatal Age (days)
Age Postpartum

assays performed in the presence and absence of AMP), the samples were directly assayed for pyrophosphatase activity. The results of the pyrophosphatase activities are presented in Table 6. A distinctive rise in pyrophosphatase activity was noted for the day 2 and day 3 samples with a high level of activity being noticed for day 3 as postulated.

UDP-Galactosyltransferase Activity Assayed in Cultured Cells

The assay for UDP-galactose : glycoprotein galactosyltransferase in cultured cells was identical to that used for pancreatic tissue. Quantity of material obtainable from tissue culture flasks precluded the preparation of membrane fractions and unless indicated otherwise all assays were done with whole cell homogenates.

Assay Requirements

Assays were run on the homogenates of the following cell lines: KB,V-79,CHO,CHO-M,Nil-8,Nil-8HSV. In all cases examined the assay requirements were similar if not identical to those previously described for the pancreatic enzyme.

Table 6

Hydrolysis of UDP-Galactose by Neonatal Rat Pancreatic Microsomes

Neonate Age[a]	% UDP-Galactose Hydrolyzed[b]		
	0 min	15 min	30 min
Day 1	0	0	2
Day 2	0	3	7
Day 3	0	8	13
Adult	0	0	2

[a]Date of parturition is day 1

[b]Hydrolysis was measured in a standard reaction mix containing 2.1 mM UDP-galactose. Description of assays is in Methods.

Cell Cycle Dependence

Glycosyltransferases have been reported to vary as a function of the cell cycle(163,164). In order to examine glycoprotein galactosyltransferase activity as a function of the cell cycle KB cells were synchronized by a double thymidine block and galactosyltransferase activity assayed at two hour time intervals for a complete cycle (24 hours). The results are shown in Figure 21. The incorporation of tritiated thymidine into a distint peak confirms synchronization. No significant variation of galactosyltransferase activity occurred.

Monosaccharides as Exogenous Acceptors

Experiments were performed to determine if the membrane galactosyltransferase from cells in culture could utilize free glucose or free N-acetylglucosamine as substrate acceptors. This would constitute a difference from the pancreatic enzyme as no transfer to free N-acetylglucosamine occurred with enzyme from this tissue. This reaction is illustrated in Figure 22. The results of the experiments are summarized in Table 7. Clearly transfer to both glucose and N-acetylglucosamine occurred, with N-acetylglucosamine being the preferred acceptor by over a factor of four.

Figure 21. Glycoprotein Galactosyltransferase Activity in Synchronized KB Cells

Log-phase KB cells were synchronized by means of a double thymidine block. Synchronization was confirmed by measuring tritiated thymidine incorporation. Glycoprotein galactosyltransferase activity was assayed every two hours. Details of procedures are in Methods.

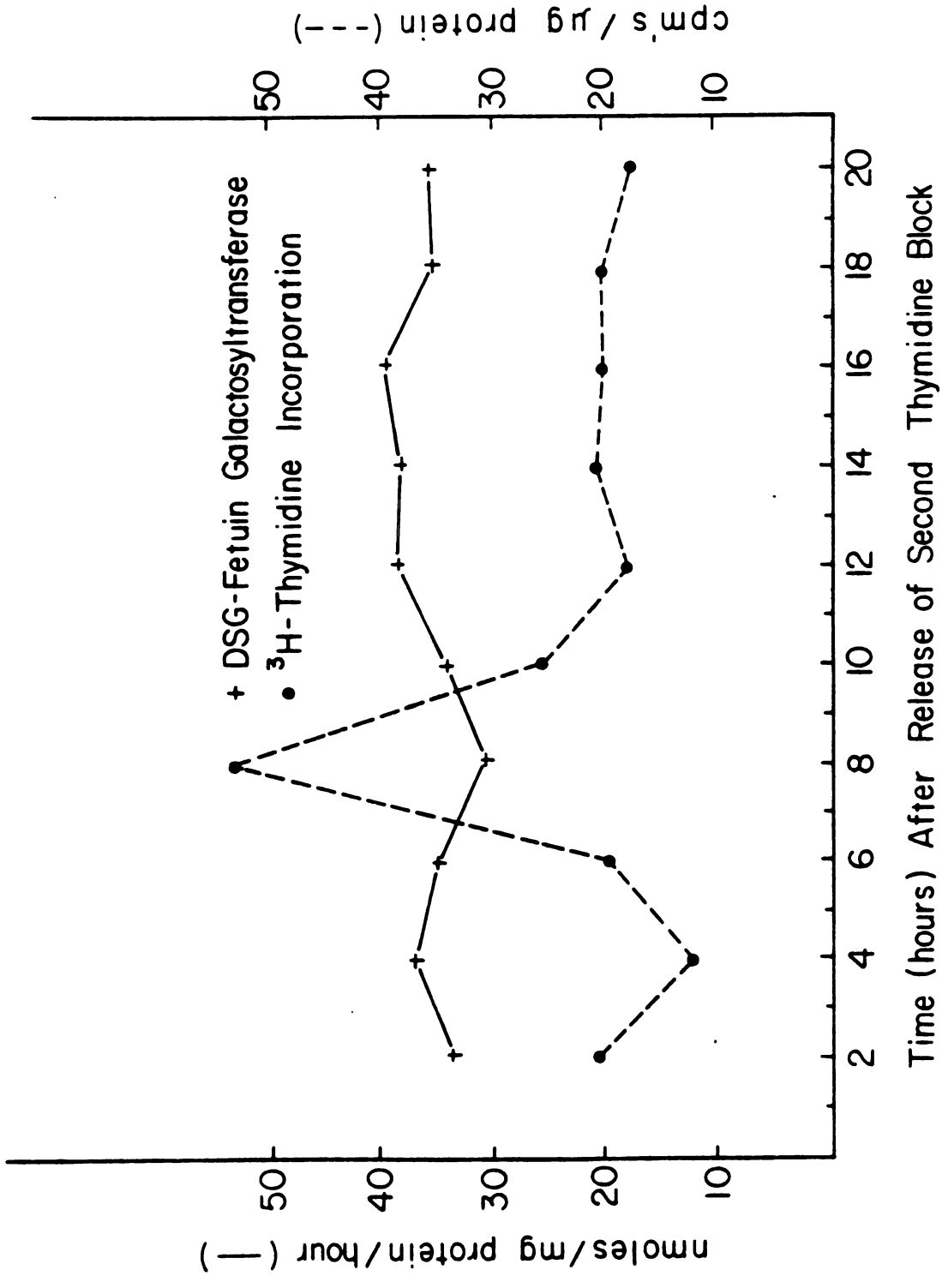

nmoles/mg protein/hour (—)
cpm's / µg protein (---)
+ DSG-Fetuin Galactosyltransferase
• ³H-Thymidine Incorporation
Time (hours) After Release of Second Thymidine Block
10
20
30
40
50
2
4
6
8
10
12
14
16
18
20

Figure 22. Lactose Synthetase (UDP-Galactose: D-Glucose 1-galactosyltransferase)

This figure illustrates the reactions catalyzed by lactose synthetase, The enzyme will utilize N-acetyl-glucosamine to form N-acetyllactosamine, however in the presence of a modifier protein, alpha-lactalbumin, the K_m for transfer to glucose is lowered and synthesis of lactose is favored.

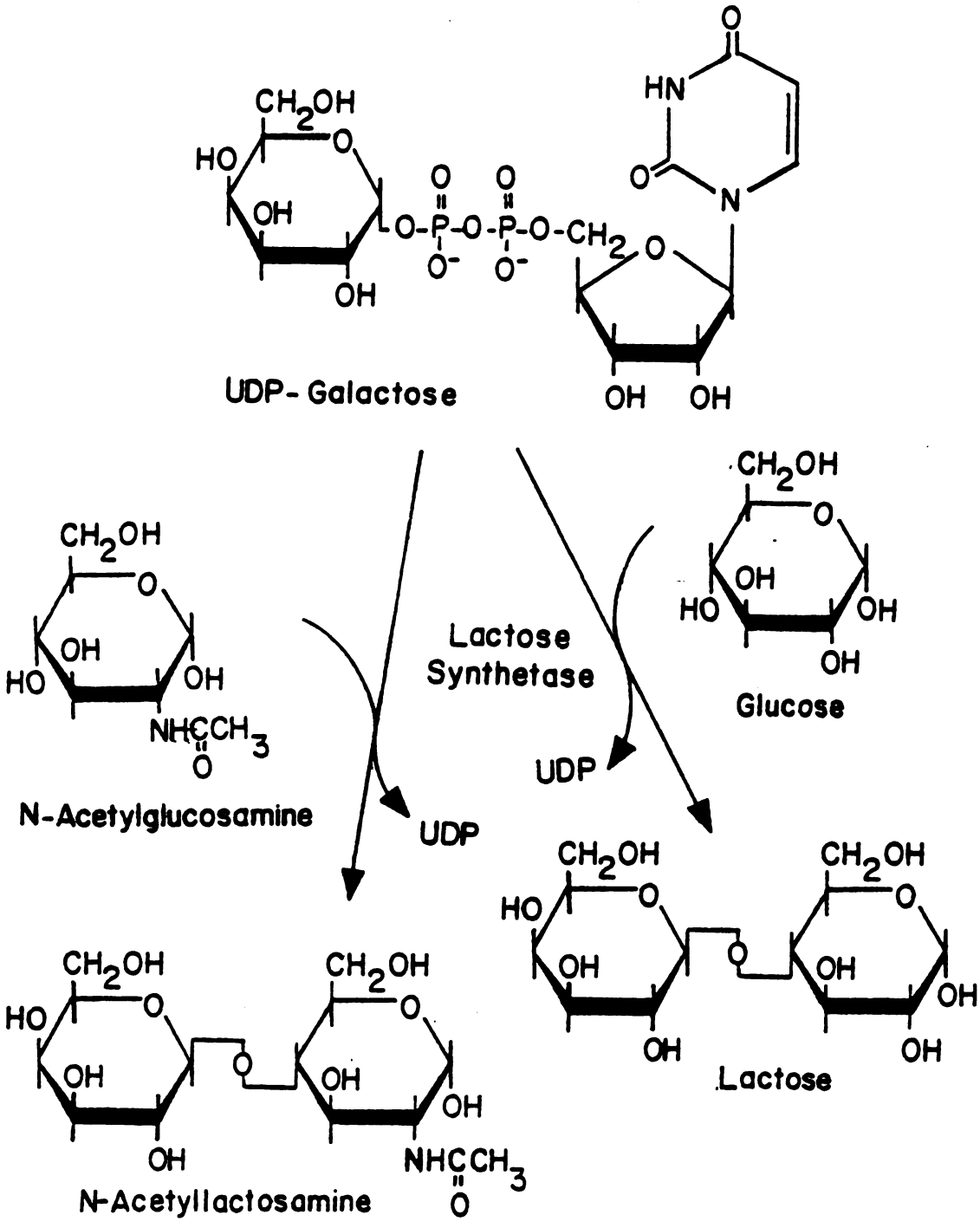

UDP- Galactose
Lactose Synthetase
Glucose
N-Acetylglucosamine
UDP
UDP
Lactose
N-Acetyllactosamine

Table 7

Galactosyltransferase Activity with Glucose and N-acetylglucosamine as Acceptors in the Presence and Absence of alpha-lactalbumin

Assay Conditions[a]	C.P.M. Eluted from Dowex-1
0 Enzyme + N-Acetylglucosamine	150
0 Enzyme + Alpha-lac	1000
0 Enzyme + Glucose + Alpha-lac	150
33 µg Enzyme + N-Acetylglucosamine	3200
33 µg Enzyme + N-Acetylglucosamine + Alpha-lac	1100
33 µg Enzyme + Glucose	750
33 µg Enzyme + Glucose + Alpha-lac	2250

[a] The assay mixture contained in 50 µl: 0.25 M sodium cacodylate buffer (pH 7.5), 10mM $MnCl_2$ and ^{14}C UDP-Galactose(5000 **d.p.m.**). Glucose and N-Acetylglucosamine when present were 50mM and alpha-lactalbumin was 7.5 µg per assay. The assay procedure is described in Methods. Enzyme source was KB cell homogenates.

Effect of Alpha-lactalbumin on Acceptor Specificity

Alpha-lactalbumin interacts specifically with many galactosyltransferases to lower the Michaelus constant for glucose to make it more suitable as an acceptor (reviewed in reference 165). This same phenomenon occurred in the cellular enzyme and is illustrated in Table 7. Alpha-lactalbumin in the reaction mixture decreased transfer to N-acetylglucosamine to base line while increasing transfer to glucose by a factor of 3.

Effect of Phorbol Ester Tumor Promoters on Galactosyltransferase Activity

The structures of the phorbol ester tumor promoters are illustrated in Figure 23 and their "in vivo" promoting activity is listed in Table 8.

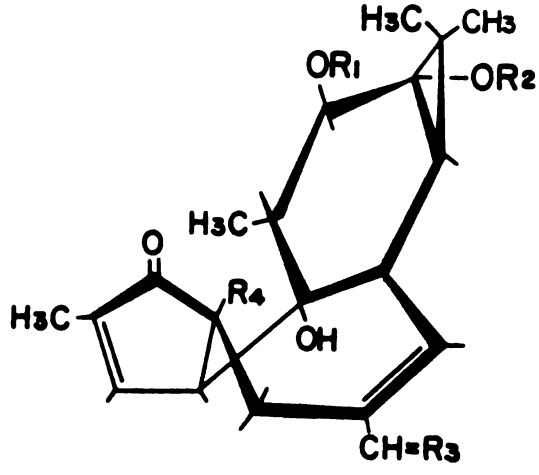
Nil-8 Cells

After exposure to TPA, phorbol retinoic acid, and retinoic acid plus TPA for 12 hours Nil-8 cells were harvested, homogenized, and assayed for galactosyltransferase activity. Results are shown in Figure 24. The glycolipid sialyltransferase data were supplied by Dr. J. Moskal. Phorbol alone gave a rise in galactosyltransferase activity while the other agents gave activities that were similar to the control value. The glycolipid sialyltransferase data deviated significantly from the glycoprotein

Figure 23. Structures of Phorbol Ester Tumor Promoters

The chemical structures and abbreviations of the phorbol ester tumor promoters used in this investigation are illustrated.

PHORBOL ESTERS



PRODUCT	ABB.	R1	R2	R3	R4
4 alpha - Phorbol 12,13-didecanoate	4 alpha-PDD	$C_9H_{19}CO$-	$C_9H_{19}C_0$-	H, OH	H
Phorbol 12,13-diacetate	PDA	CH_3CO-	CH_3CO-	H, OH	H
Phorbol 4-O-methyl 12-myristate-13-acetate	MPMA	$C_{13}H_{27}CO$-	CH_3CO-	H, OH	CH_3
Phorbol 12,13-dibutyrate	PDB	C_3H_7CO-	C_3H_7CO	H, OH	H
Phorbol 12,13-didecanoate	PDD	$C_9H_{19}CO$-	$C_9H_{19}CO$-	H, OH	H
Phorbol 12-myristate-13-acetate	PMML	$C_{13}H_{27}CO$-	CH_3CO-	H, OH	H

Table 8

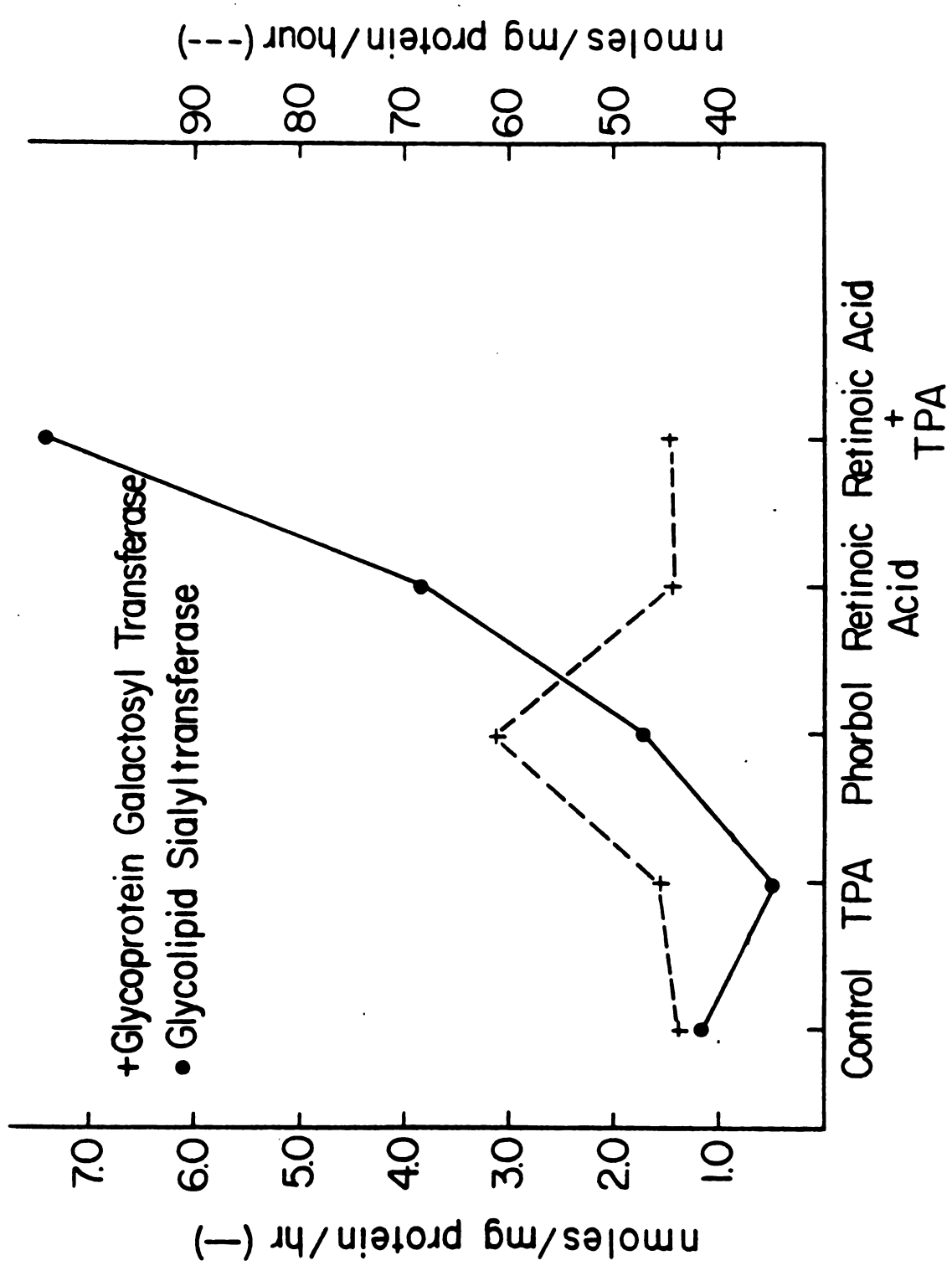
Phorbol Ester Tumor Promoters

Phorbol Analogue	Abbreviation	In Vivo Tumor Promoting Activity [a]
Phorbol	Phor	-
4alpha-phorbol 12,13-didecanoate	4αPDD	-
Phorbol 12,13-diacetate	PDA	+
4-O-methylphorbol 12-myristate 13-acetate	MPMA	+
Phorbol 12,13-dibutyrate	PDB	++
Phorbol 12,13-didecanoate	PDD	+++
Phorbol 12-myristate 13-acetate	PMML	++++

[a]Yotti,L.P., Chang,C.C., and Trosko, J.E. Science 206,1089 (1979)

Figure 24. The Effect of TPA and Retinoic Acid on Glycoprotein Galactosyltransferase Activity in Nil-8 Cells

Log-phase Nil-8 cells were grown in media containing the specified chemical (10 ng/ml) for 12 hours. After this exposure the media was removed and the cells were washed three times with PBS. The cells were then harvested by scrapping with a rubber policeman. The T-flasks were then rinsed in PBS and the cells collected by centrifugation. Following homogenization galactosyltransferase activity was assayed as described in Methods. The glycolipid sialytransferase utilized lactosylceramide as an acceptor (data provided by Dr. J. Moskal).


nmoles/mg protein/hr (—)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
nmoles/mg protein/hour (---)
90
80
70
60
50
40
+Glycoprotein Galactosyl Transferase
• Glycolipid Sialyltransferase
Control
TPA
Phorbol
Retinoic Acid
Retinoic Acid + TPA

galactosyltransferase activity in that sialic acid transfer to glycolipid was elevated by retinoic acid and elevated synergistically by retinoic acid plus TPA.

Nil-8HSV Cells

Experiments identical to those performed on Nil-8 cells were done with a tranformed cell line Nil-8HSV. The results are illustrated in Figure 25. TPA and phorbol stimulated enzyme activity to approximately the same extent. Retinoic acid stimulated activity over that observed with TPA. No synergistic effect was seen with retinoic acid plus TPA. The glycolipid sialyltransferase activity interestingly followed the same trend as did the glycoprotein galactosyltransferase.

KB Cells

Similar studies were performed with KB cells as were done with the Nil-8 and Nil-8HSV cells. Results are illustrated in Figure 26. Phorbol elevated galactosyltransferase activity but to a lesser extent than did TPA. Retinoic acid elevated the activity to a greater extent. As in the Nil-8HSV cells the glycolipid sialyltransferase activity paralleled the glycoprotein galactosyltransferase activity. A pronounced negative synergistic effect was noticed with retinoic acid plus TPA. Addition of epidermal growth factor (EGF) gave a lower than control level of galactosyltransferase activity and EGF inhibited the

Figure 25. The Effect of TPA and Retinoic Acid on Glycoprotein Galactosyltransferase Activity in Nil-8HSV Cells

Log-phase Nil-8HSV cells were assayed for glycoprotein galactosyltransferase activity after exposure to the listed agents in an identical mannor to that described for Nil-8 cells (see Figure 24).

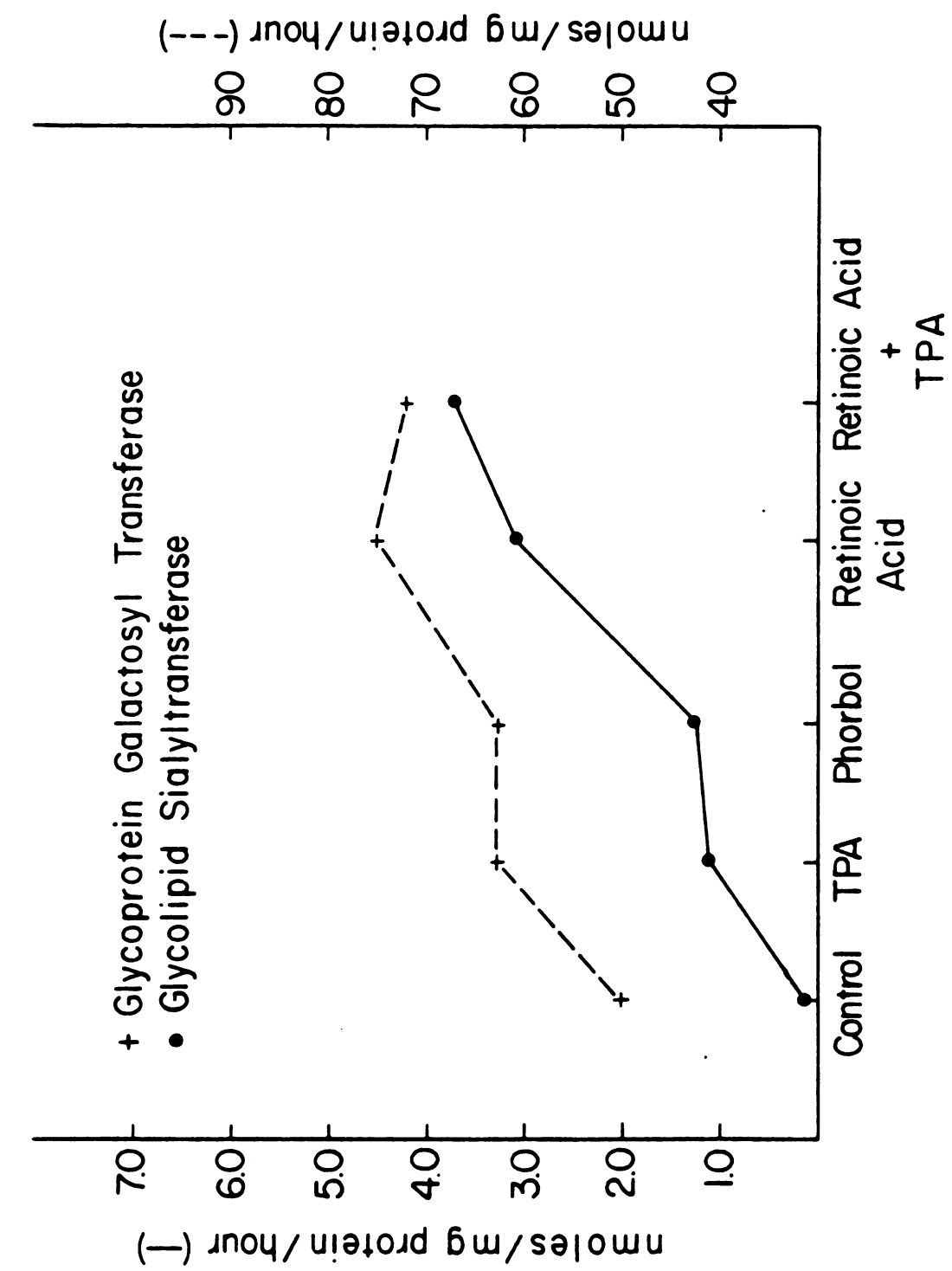
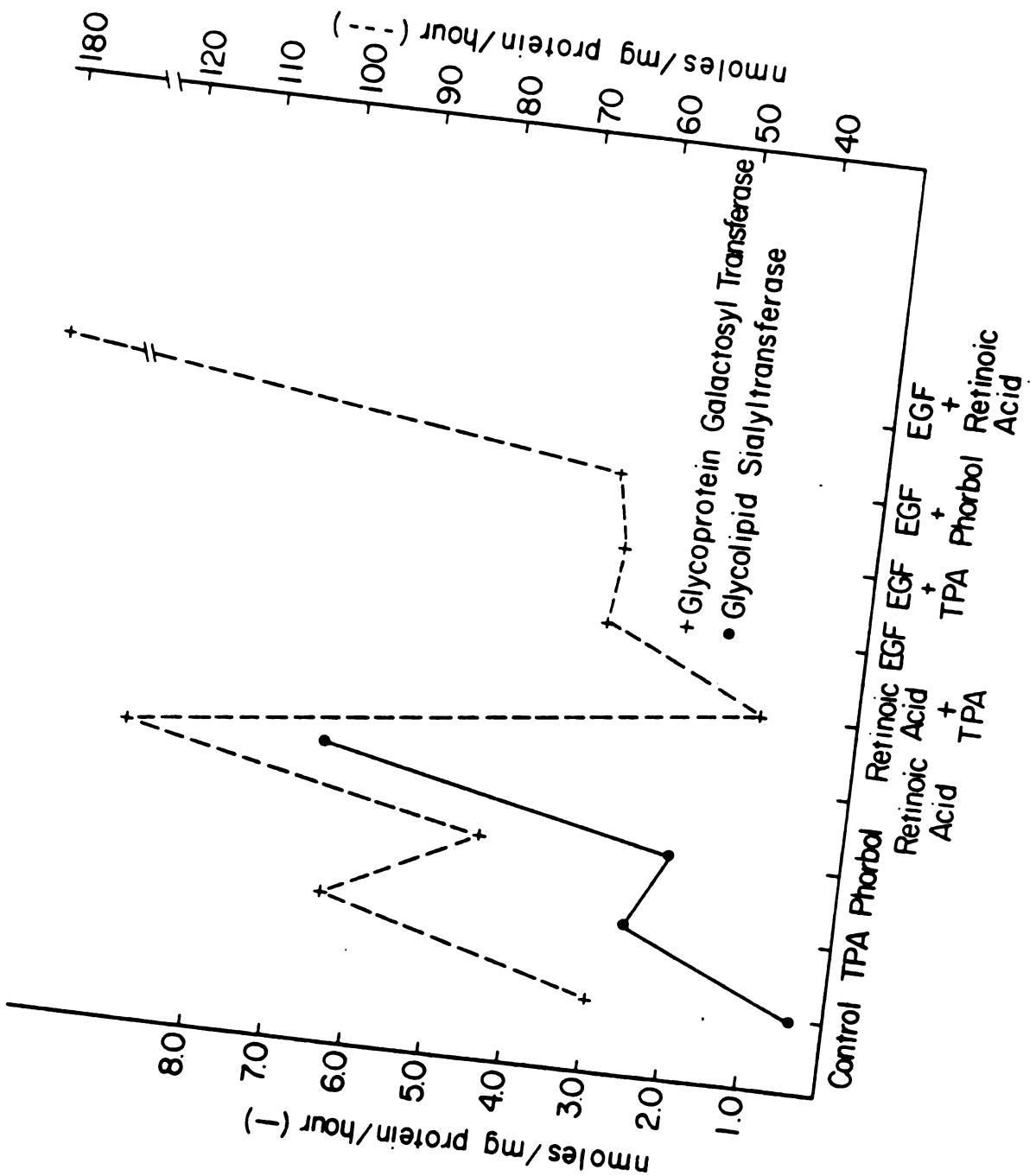

nmoles/mg protein/hour (—)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
nmoles/mg protein/hour (---)
90
80
70
60
50
40
+ Glycoprotein Galactosyl Transferase
• Glycolipid Sialyltransferase
Control
TPA
Phorbol
Retinoic Acid
Retinoic Acid + TPA

Figure 26. The Effect of TPA and Retinoic Acid on Glycoprotein Galactosyltransferase Activity in KB Cells

Log-phase KB cells were assayed for glycoprotein galactosyltransferase activity after exposure to the listed agents in as identical mannor to that described for Nil-8 cells(see Figure 24). Epidermal growth factor (EGF) was present at a concentration of 20 ng/ml.


nmoles/mg protein/hour (—)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
nmoles/mg protein/hour (---)
180
120
110
100
90
80
70
60
50
40
+ Glycoprotein Galactosyl Transferase
• Glycolipid Sialyltransferase
Control
TPA Phorbol
Retinoic Acid
Retinoic Acid + TPA
EGF
EGF + TPA
EGF + Phorbol
EGF + Retinoic Acid

elevation seen with TPA and phorbol. A pronounced positive synergistic effect was seen with EGF plus retinoic acid.

Effect of Cell Density on Tumor Promoter Alterations in Glycoprotein Galactosyltransferase Activity

The effect of cell density on glycoprotein galactosyltransferase alterations by tumor promoters is shown in Figure 27. Two cell lines were used in these studies: KB and V-79 cells. The promoters were added when the cells were in either log-phase or confluent growth. Clearly no difference was noted between activities in confluent versus log phase cells. Mellitin , a polypeptide promoter, reacted similarly to TPA with no significant difference between confluent and log-phase cells.

Effect of Tumor Promoters on Glycoprotein Galactosyltransferase Activity in CHO and a Membrane Mutant CHO Cell Lines

The effects of TPA,retinoate, and butyrate on glycoprotein galactosyltransferase activity were examined in CHO and a membrane mutant CHO cell line (CHO-M). Results are shown in Figure 28. TPA significantly elevated activity in CHO-M but not in CHO cells. Retnoate and butyrate significantly elevated activity in both cell lines with a 4 fold increase being seen in CHO-M cells and a 2-fold increase in CHO cells.

Figure 27. The Effect of Cell Density on Tumor Promoter Induced Alterations in a Glycoprotein Galactosyltransferase

Promoters were added to V-79 or KB cells growing in monolayers when the cells were log-phase (50% confluent) or confluent. Cells were harvested and assayed for galactosyltransferase activity as described in Methods.

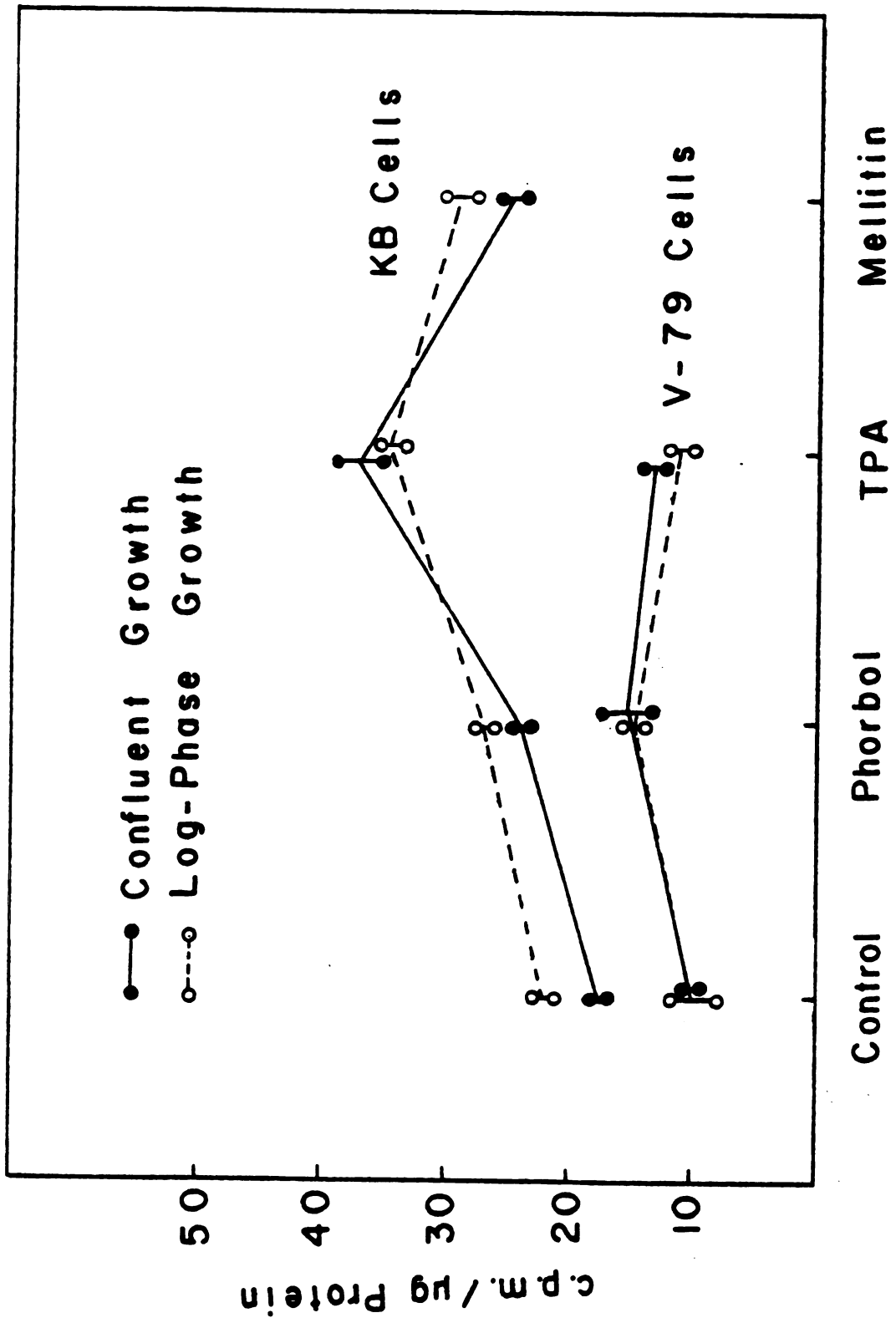
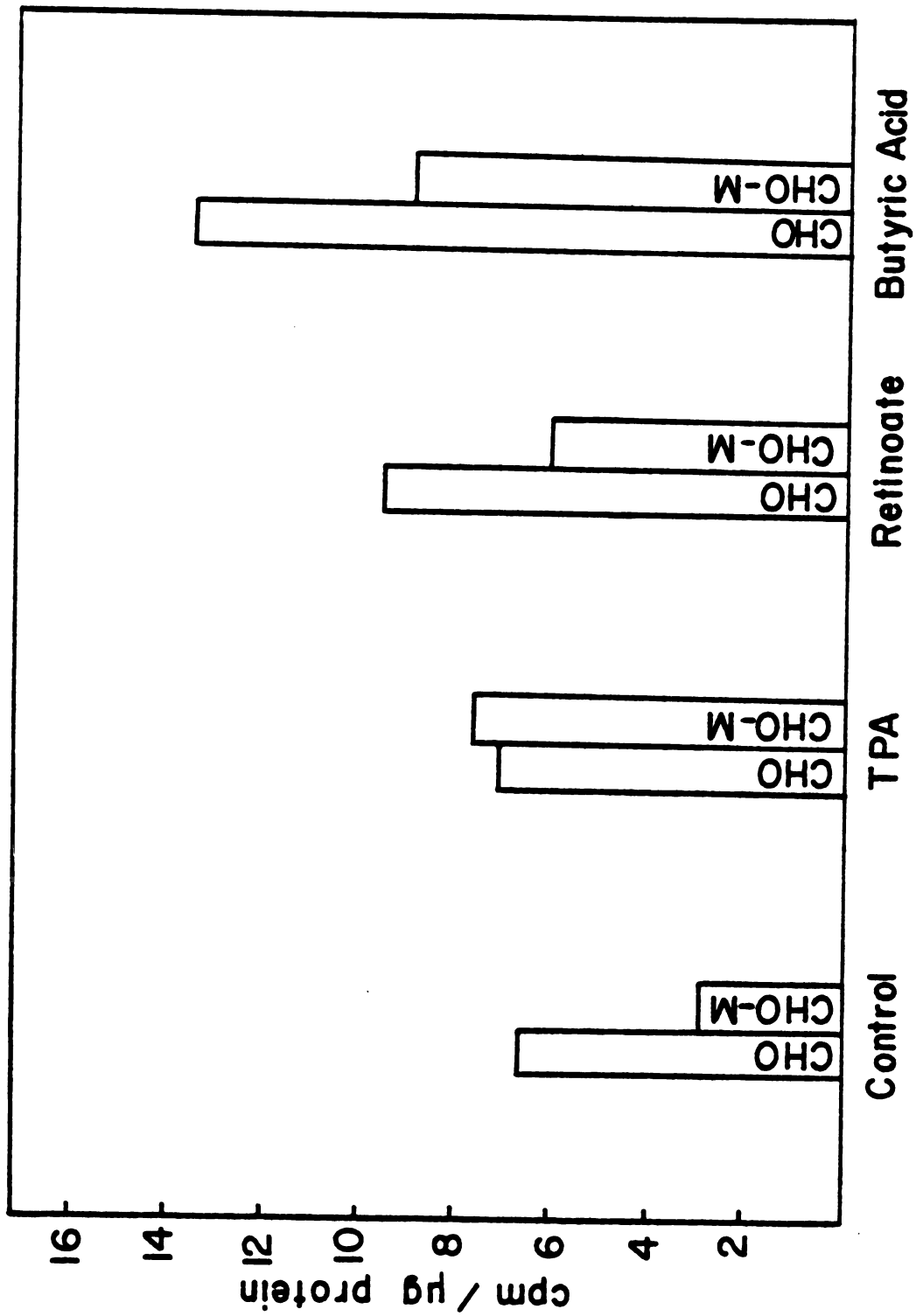

c.p.m. / µg Protein
50
40
30
20
10
Control
Phorbol
TPA
Mellitin
KB Cells
V-79 Cells
Confluent Growth
Log-Phase Growth

Figure 28. The Effect of TPA, Retinoate, and Butyric Acid on a Glycoprotein Galactosyltransferase Activity in CHO and CHO-M Cells

Log-phase cells were exposed to the chemical agents (TPA-10 ng/ml, Retinoate-100 ng/ml, and butyric acid- 0.16mM) as described in Methods. After harvesting and homogenization standard galactosyltransferase assays were performed.


cpm / µg protein
2
4
6
8
10
12
14
16
Control
CHO
CHO-M
TPA
CHO
CHO-M
Retinoate
CHO
CHO-M
Butyric Acid
CHO
CHO-M

Graded Phorbol Ester Series

A graded series of phorbol ester tumor promoters was used to determine if "in vivo" promoter activity could be related to the level of glycoprotein galactosyltransferase activity in either CHO or CHO-M cells. Results are shown in Figure 29. No definite trend was seen in either CHO or CHO-M cells. CHO cells were relatively unresponsive to the phorbol esters while the CHO-M cells showed elevated activity in most instances.

Phosphorylation Assays in CHO and CHO-M Plasma Membrane Fractions

Purification of Plasma Membrane Fractions

Cells were grown is suspension culture, harvested and a plasma membrane fraction prepared by the method of Thom et al. (157) as outlined in Figure 30. Transmission electron microscopy of the preparations showed vesicles, smooth membranes, and the general membranous character of the preparation.

Assay System

The phosphorylation assay followed the procedure of Carpenter et al. (166). Details are presented in Methods. Conditions for the assay were a four minute incubation at 4 degrees C (Figure 31). The assay was linear with 10 to 60 micrograms of protein (Figure 32).

Figure 29. The Effect of Phorbol Ester Tumor Promoters on a Glycoprotein Galactosyltransferase Activity in CHO and CHO-M Cells

Phorbol ester tumor promoters were added to log-phase CHO and CHO-M cells at 10 ng/ml. After a 12 hour exposure cells were harvested and glycoprotein galactosyltransferase activity was determined. Results are expressed as the per cent difference from the control study.

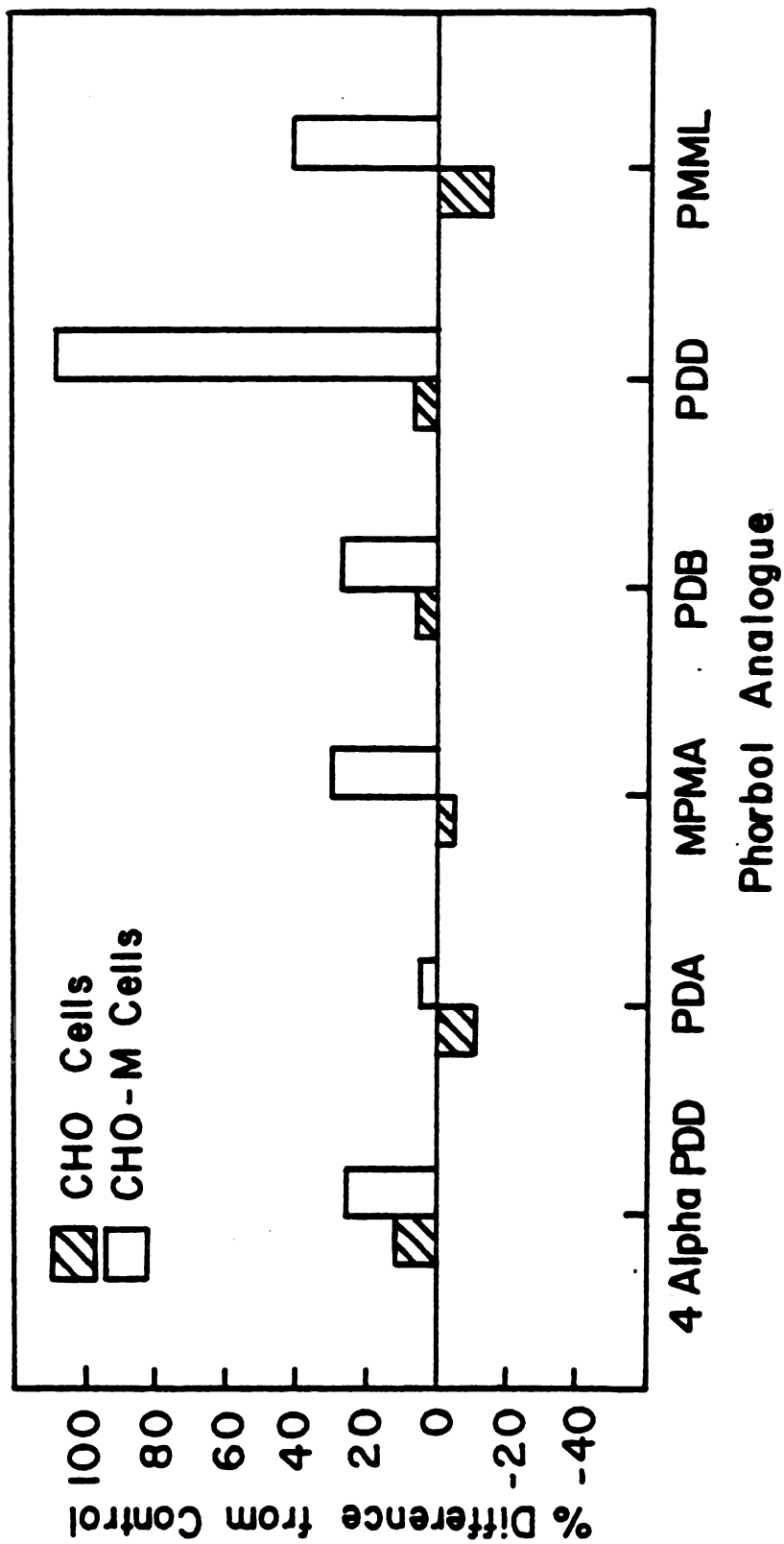

% Difference from Control
100
80
60
40
20
0
-20
-40
CHO Cells
CHO-M Cells
4 Alpha PDD
PDA
MPMA
PDB
PDD
PMML
Phorbol Analogue

Figure 30. Purification of a Plasma Membrane Fraction from Cultured Cells

Plasma membrane fractions were purified from whole cell pellets by the procedure of Thom et al.(215). The outline of the procedure is given in the figure. Details are described in Methods.

PURIFICATION OF PLASMA MEMBRANE FRACTION

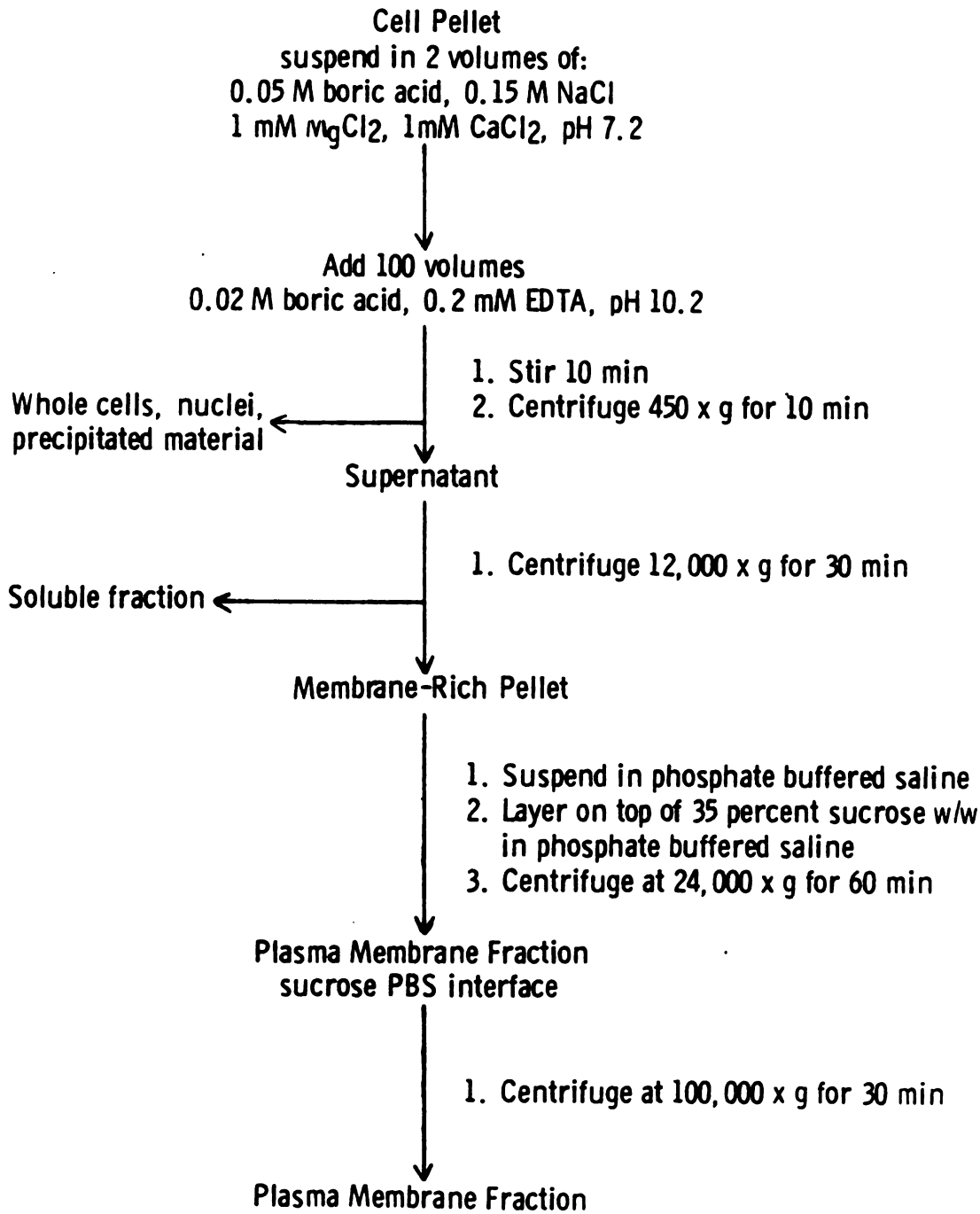


Figure 31. The Rate of Endogenous Phosphorylation as a Function of Temperature

Standard phosphorylation assays were run at 37 degrees C and at 4 degrees C with 50 µg of KB cell homogenate. Assays were terminated at the incubation times indicated and the incorporation into acid insoluble material determined. Assay details are in Methods.

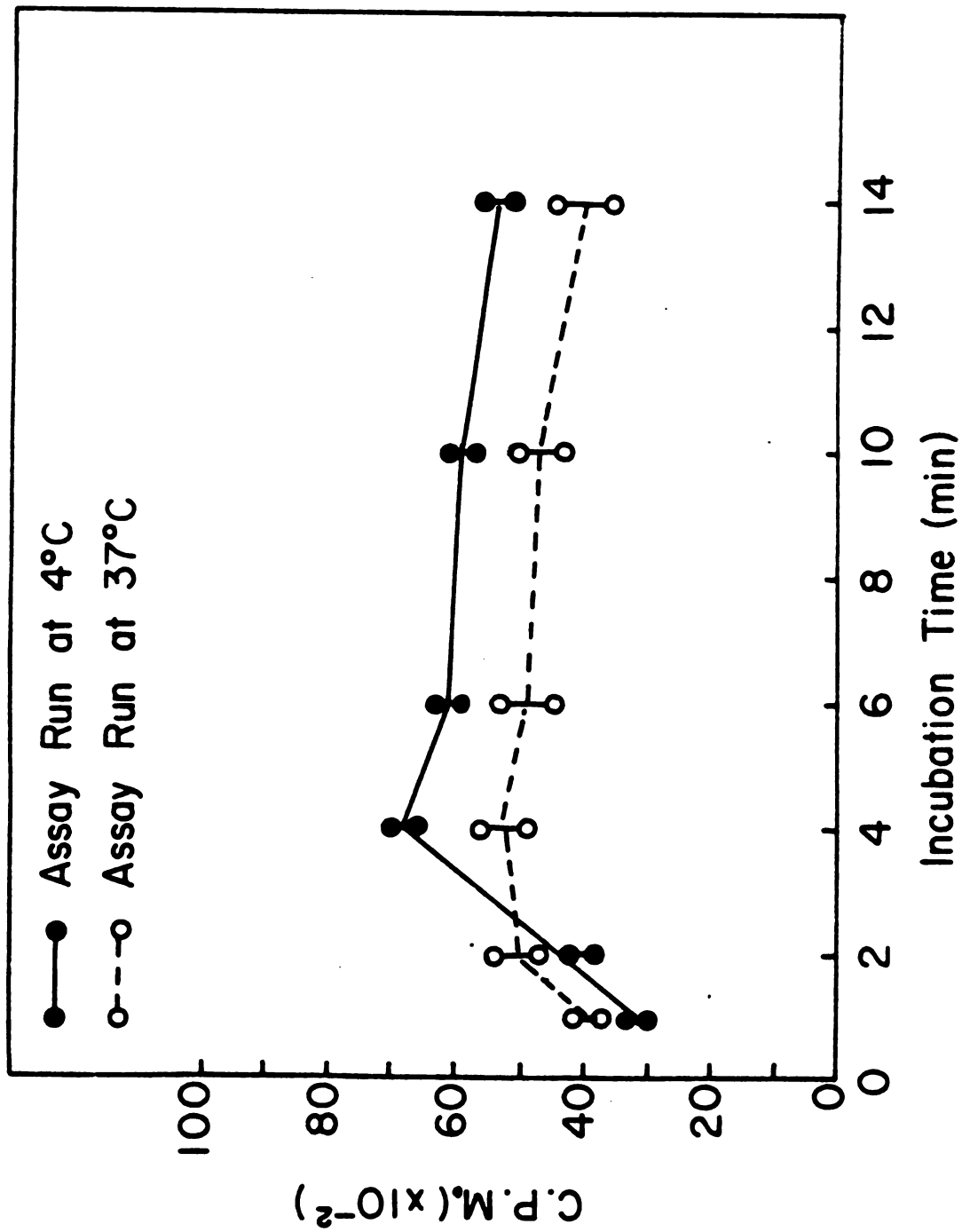
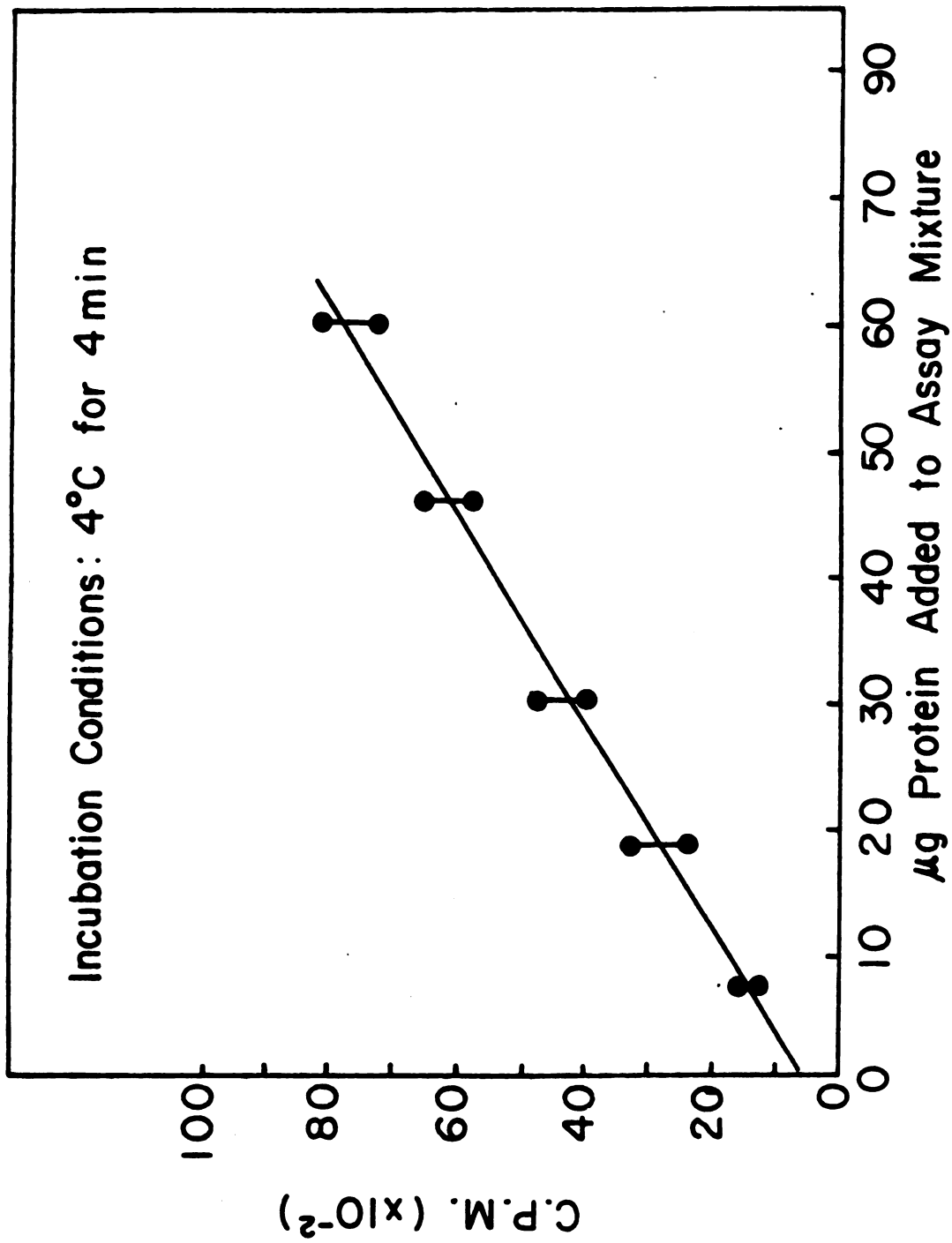

Assay Run at 4°C
Assay Run at 37°C
C.P.M.(x10⁻²)
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Incubation Time (min)

Figure 32. Linearity of the Phosphorylation Reaction with Respect to Enzyme Concentration

This figure demonstrates the linearity of the endogenous phosphorylation assay with respect to protein added. Enzyme source was KB cell homogenates. Incubation conditions were a 4 minute incubation at 4 degrees C. Assays were initiated by addition of labeled substrate (^{32}P-ATP). Details of assay are in Methods.


Incubation Conditions: 4°C for 4 min
C.P.M. (x10⁻²)
0
20
40
60
80
100
0
10
20
30
40
50
60
70
80
90
μg Protein Added to Assay Mixture

Endogenous Phosphorylation

CHO Plasma Membrane Phosphorylation - The phosphorylation data for CHO plasma membrane preparations are illustrated in Figure 33. No sizable alterations of phosphorylation were noticed with any promoter tested.

CHO-M Plasma Membrane Phosphorylation -Distinct alterations in endogenous phosphorylation were obtained with CHO-M plasma membrane preparations (Figure 34). A uniform depression to approximately 30 percent was seen with all the phorbol esters tested except for PDA where a two fold induction in activity was noted.

KB Plasma Membrane Phosphorylation - The patterns of endogenous phosphorylation for KB cell plasma membranes is illustrated in Figure 35. The pattern is analogous to CHO cells in that alterations were obtained with few of the promoters. As with the CHO-M cells an increase occurred with the promoter PDA. At least with respect to endogenous phosphorylation KB cells have membrane properties comparable in some aspects to CHO cells and in some respects to CHO-M cells.

Effect of Butyrate on Endogenous Membrane Phosphorylation - The effect of butyrate on endogenous phosphorylation in KB, CHO, and CHO-M cells is illustrated in Figure 36. Clear differences are noted. Butyrate had little effect on phosphorylation in KB and CHO-M cells; however, a clear stimulatory effect was seen in CHO cells. CHO-M cells had a

Figure 33. Alteration of Endogenous Phosphorylation in CHO Plasma Membrane Preparations by Phorbol Ester Tumor Promoters

Standard phosphorylation assays were run with 10 µg of CHO plasma membrane fraction per assay. Promoters (10 ng/ml) were added to the complete reaction mixture minus ATP. The assay mixture was then incubated for 2 minutes at 37 degrees C, and then returned to an ice bath where the reaction was initiated by the addition of ATP. Reactions were terminated after 4 minutes and radioactive incorporation into acid insoluble material was determined by liquid scintillation spectrometry.

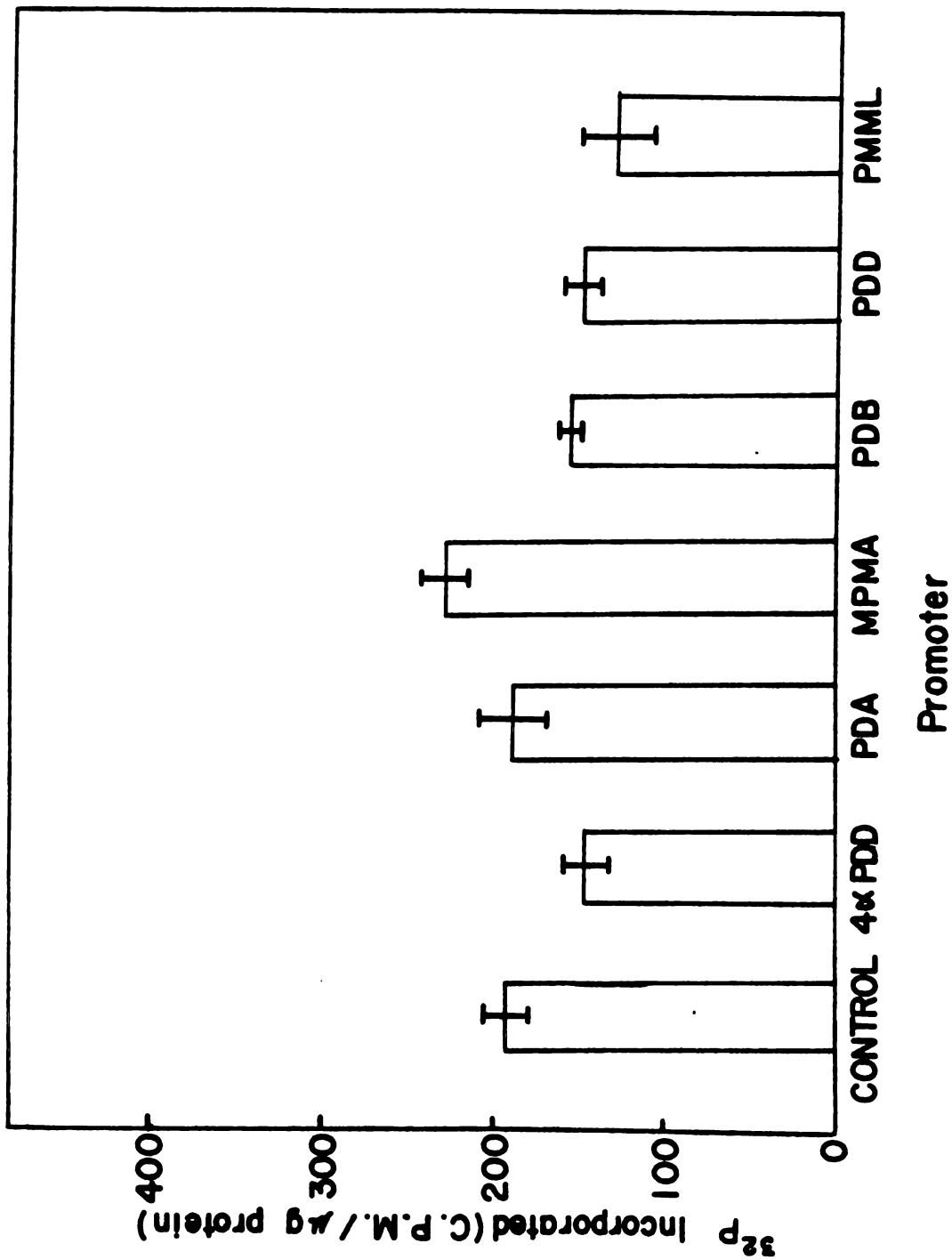

400
300
200
100
0
^{32}P Incorporated (C. P.M. / µg protein)
CONTROL
4α PDD
PDA
MPMA
PDB
PDD
PMML
Promoter

Figure 34. Alteration of Endogenous Phosphorylation in CHO-M Plasma Membrane Preparations by Phorbol Ester Tumor Promoters

Standard phosphorylation assays were run with 10 µg of CHO-M plasma membrane fraction per assay. Details are in Figure legend 33.

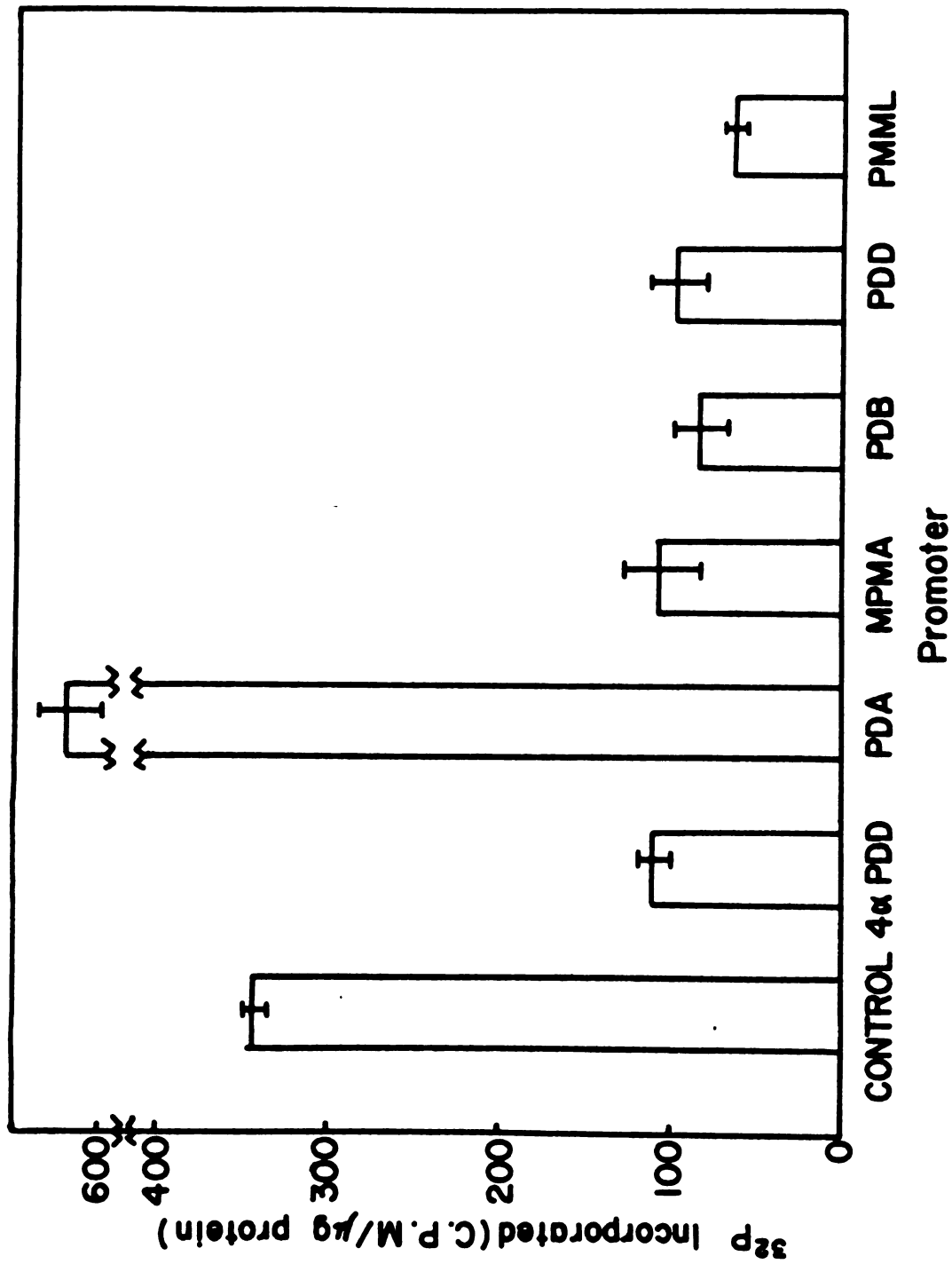

600
400
300
200
100
0
32P Incorporated (C.P.M/μg protein)
CONTROL
4α PDD
PDA
MPMA
PDB
PDD
PMML
Promoter

Figure 35. **Alteration of Endogenous Phosphorylation in KB Plasma Membrane Preparations by Phorbol Ester Tumor Promoters**

Standard phosphorylation assays were run with 10 µg of KB plasma membrane fraction per assay. Details are in Figure legend 33.

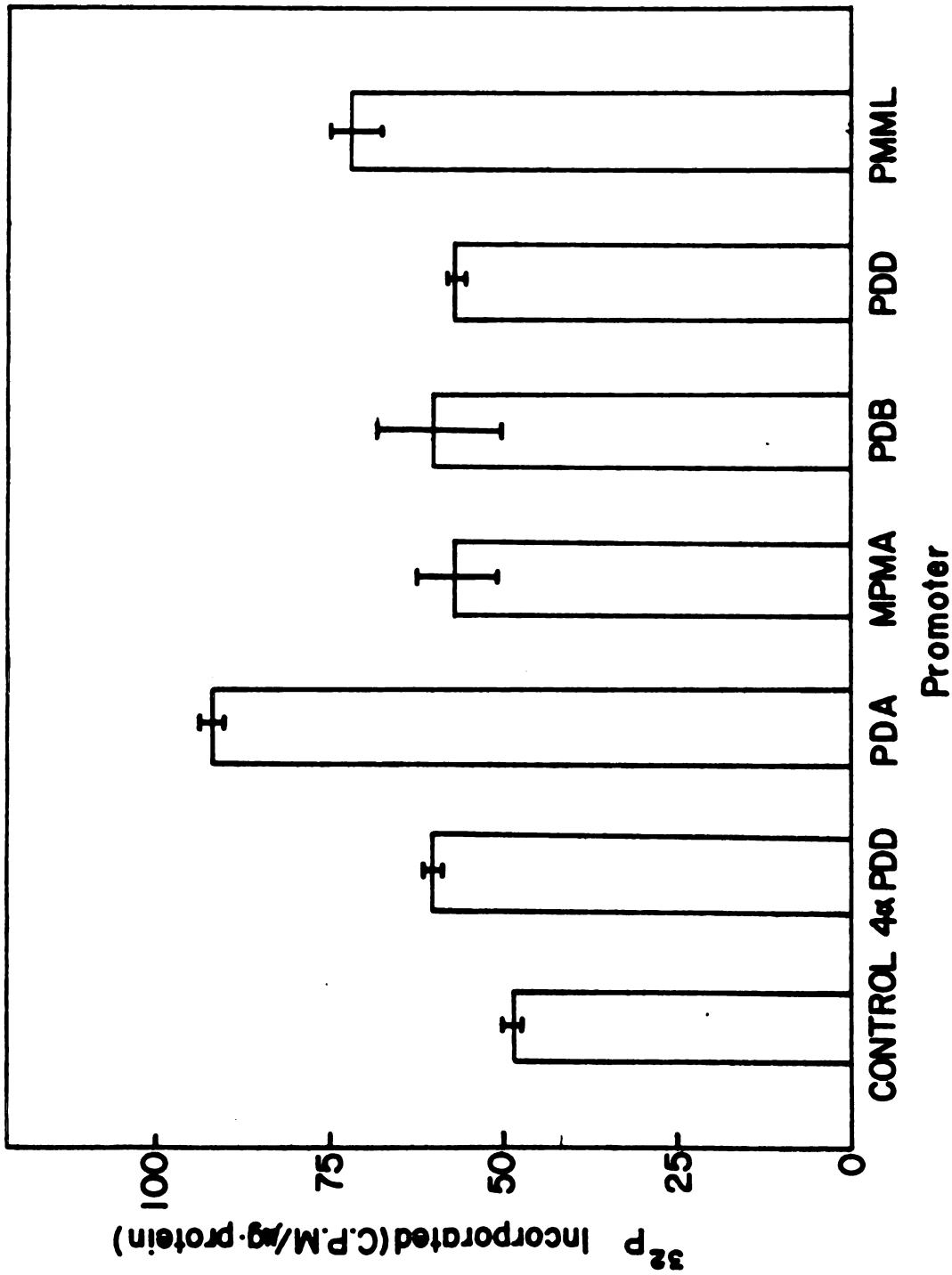
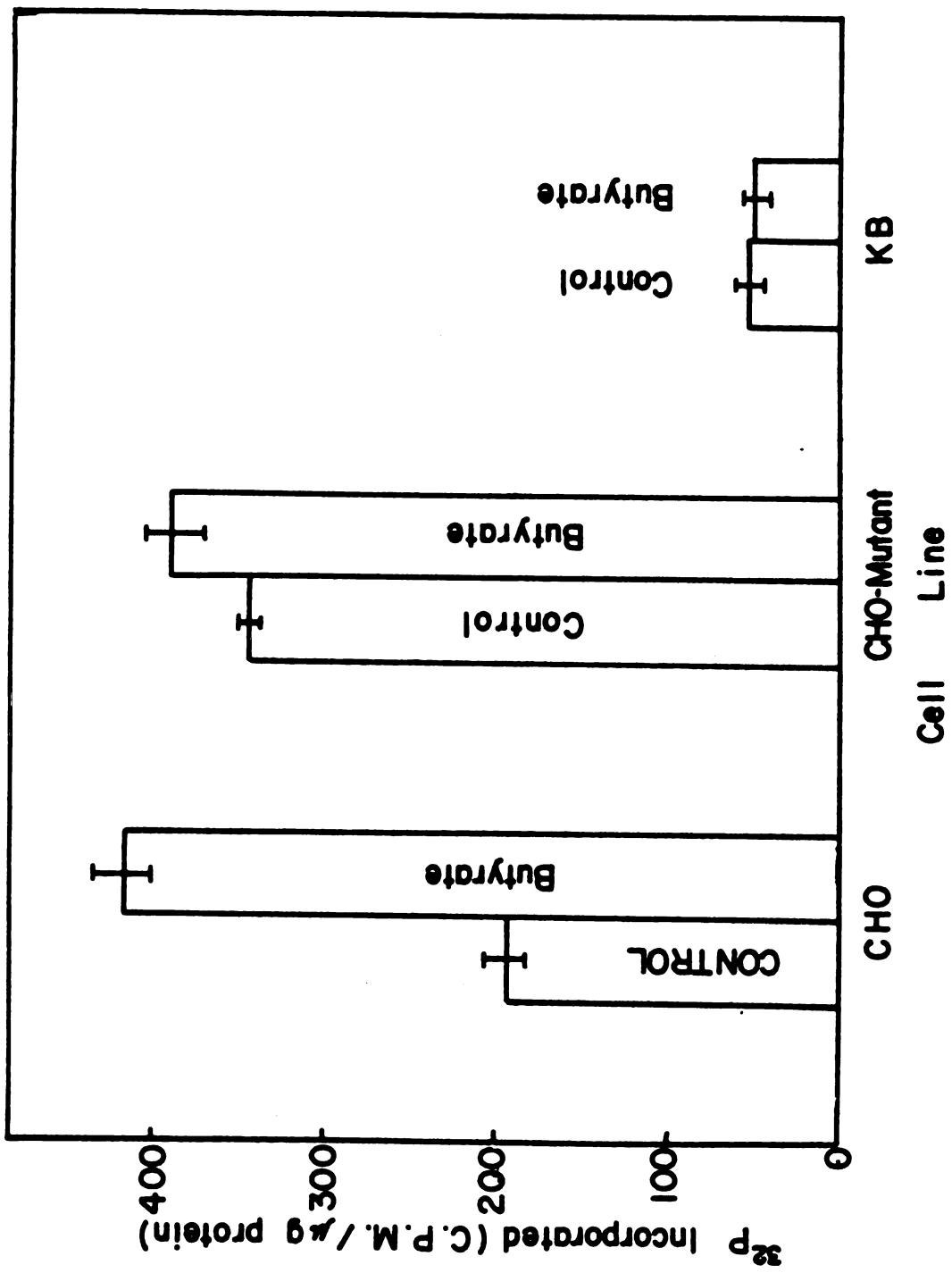

^{32}P Incorporated (C.P.M./μg·protein)
0
25
50
75
100
CONTROL
4α PDD
PDA
MPMA
PDB
PDD
PMML
Promoter

Figure 36. Alteration of Endogenous Phosphorylation in CHO, CHO-M, and KB Plasma Membrane Preparations by Butyrate

Standard phosphorylation assays were run with 10 μg of plasma membrane protein per assay. Log-phase cells were exposed to 0.16 mM butyrate for 12 hours before harvesting. Details of procedures are in Methods.


^{32}P Incorporated (C.P.M. / μg protein)
0
100
200
300
400
CONTROL
Butyrate
Control
Butyrate
Control
Butyrate
CHO
CHO-Mutant
KB
Cell Line

higher endogenous level of phosphorylation than do CHO cells; however, in the presence of butyrate both had similar levels of incorporation.

Autoradiograms of Assay Mixtures Run on Polyacrylamide Gels

To determine whether alterations of specific protein phosphorylation would occur in the presence of the different tumor promoters, whole cell reaction mixtures were run on SDS · polyacramide gels. When stained for protein by Coomassie blue numerous bands were noted but no clear differences were noted with any promoter treatment (Figure 37).

Autoradiograms of the gels revealed distinct differences between CHO and CHO-M cells with different promoters (Figures 38,39). There was significantly greater incorporation into CHO-M cells than CHO cells (Figures 38,39 lanes 11 and 1). Cells grown in the presence of butyrate for 12 hours showed significantly greater incorporation than control cells (lane 9 vs. lane 17) In CHO cells little additional incorporation occurred when PDA was added exogenously to the system (lane 1). However, a large increase was obtained when cells were grown in its presence (lane 5). A similar but smaller effect was noted in CHO-M cells (lane 16 and lane 20).

Figure 37. SDS-Polyacrylamide Gel of CHO and CHO-M Cell Homogenates Exposed to Tumor Promoters and Anti-Promoters

Cell Line	Chemical Effector				
	Retinoate	Butyrate	PDD	PDA	PMML
CHO-M	lane 1	lane 3	lane 5	lane 7	lane 9
CHO	lane 2	lane 4	lane 6	lane 8	lane 10

Log-phase cells were exposed to the chemical effector for 12 hours and harvested as normally done for enzymatic assays. After homogenization 50 µg of protein was added to 50 µl of sample buffer, boiled for 3 minutes in a boiling water bath, and applied to a lane of a 7% SDS-polyacrylamide gel. After electrophoresis the gel was stained for protein with Coomassie Blue stain. Details of procedures are in Methods.

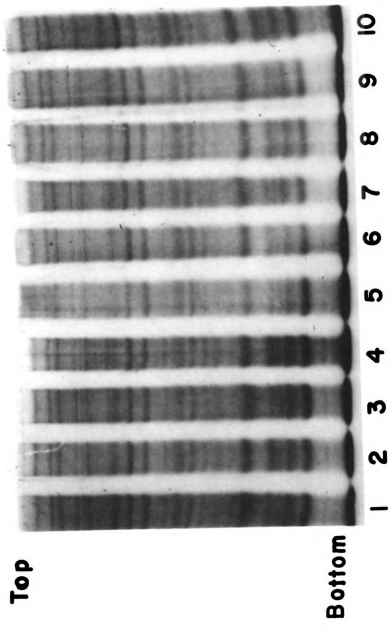
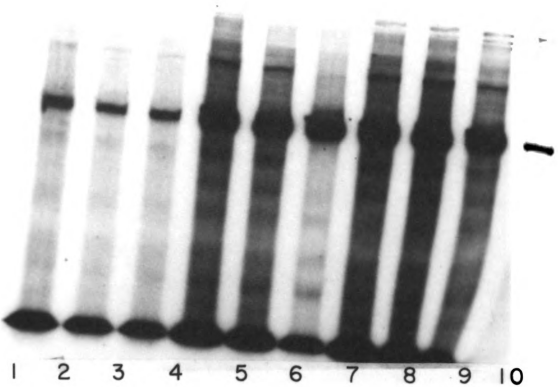

Top
Bottom
1
2
3
4
5
6
7
8
9
10

Figure 38. Autorads of Endogenous Phosphorylation Assays - CHO Cells

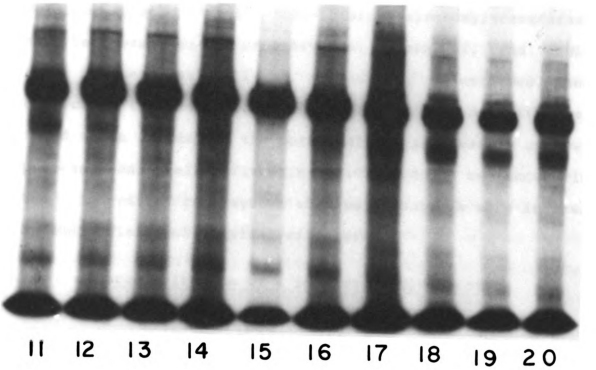
Endogenous phosphorylation assays were stopped by the addition of 50 µl of protein buffer and placing the reaction mixture in a boiling water bath for 3 minutes. The samples were then electrophoresed on 7% SDS-polyacrylamide gels. After electrophoresis the gels were dried and exposed to X-ray film for 7 days. Details of procedure are in Methods.



1 CHO control cells + exogenous PDA
2 CHO control cells + exogenous PMML
3 CHO control cells + exogenous beta-estradiol
4 CHO control cells + exogenous butyrate
5 CHO cells grown in the presence of PDA
6 CHO cells grown in the presence of TPA
7 CHO cells grown in the presence of retinoic acid
8 CHO cells grown in the presence of beta-estradiol
9 CHO cells grown in the presence of butyrate
10 CHO control cells

Figure 39. Autorads of Endogenous Phosphorylation Assays - CHO-M Cells

Details of procedures are identical to Figure legend 38.



11 CHO-M control cells
12 CHO-M cells grown in the presence of retinoic acid
13 CHO-M cells grown in the presence of butyrate
14 CHO-M cells grown in the presence of beta-estradiol
15 CHO-M cells grown in the presence of TPA
16 CHO-M cells grown in the presence of PDA
17 CHO-M control cells + exogenous butyrate
18 CHO-M control cells + exogenous beta-estradiol
19 CHO-M control cells + exogenous TPA
20 CHO-M control cells + exogenous PDA

DISCUSSION

The relative activities of a desialyzed and degalactosylated fetuin (DSG-fetuin) galactosyltransferase have been examined in the embryonic, postnatal, and adult rat pancreas as well as in normal, transformed, and chemically treated cells grown in culture. Activity was found to be induced in the postnatal rat pancreas by unknown processes and similar rises in activity could be shown in the "in vitro" cell system when perturbations were induced by the addition of chemical effectors.

Characterization of a DSG-Fetuin Glycoprotein Galactosyltransferase in the Rat Pancreas

Adult Pancreas

Characterization of a DSG-fetuin galactosyltransferase in the pancreas was carried out in order to relate its properties to a desialylzed mucin galactosyltransferase previously described in this tissue (121). A problem with the mucin transferase data is the extreme heterogeneity of the mucin substrate available. As Figure 9 demonstrates, multiple species are present, as demonstrated on SDS polyacrylamide gels. The exact nature of the recipient substrate is unclear. In contrast, fetuin, purchased from GIBCO, is clearly more homogeneous (Figure 9) and transfer to DSG-fetuin was found to be localized only in the major protein peak (data not shown).

Fetuin, a glycoprotein of fetal calf serum, has served as a model compound for studies of the biosynthesis and biological roles of glycoproteins. Its oligosaccharide moieties are 23 percent of its molecular weight and contain: galactose, mannose, N-acetylglucosamine, N-acetylgalactosamine, and sialic acids. Fetuin contains three asparagine-linked oligosaccharides (80 percent of carbohydrate weight) and three O-glycosidically linked oligosaccharides attached to one threonine and two serine residues. Consequently, galactosyltransferase activity with fetuin as a substrate will potentially be measuring incorporation into both GlcNac (asparagine type) and GalNac (serine-threonine type) type oligosaccharides. The relative incorporation into N-glycosidic versus O-glycosidic type linkages can be estimated by exploiting the base-catalyzed beta-elimination reactions that the serine (threonine)-linked sugars undergo (167). Results with the galactosyltransferase assay system. indicate that 90 percent of bound labeled galactose is stable to conditions which favor beta-elimination. Consequently, it is concluded that when DSG-fetuin is utilized as an acceptor the predominant measured activity is to the "complex type" oligosaccharides linked to asparagine residues.

An important question to be addressed was if differences in galactosyltransferase activity would be found between an acceptor of the serine (threonine) type oligosaccharide (mucin) and the potentially more interesting

complex-type asparagine oligosaccharide (fetuin). It should be noted that these enzymatic activities are quite probably distinct. The mucin acceptor ends in N-acetylgalactosamine while the fetuin complex acceptor ends in N-acetylglucosamine and current ideas would ascribe two specific enzymatic activities for these substrate transfers (31).

The galactosyltransferase specificities in Table 2 indicate that mucin is an excellent substrate for the galactosyltransferase enzyme. Microheterogeneity of the carbohydrate chains of mucin occurs and these data support the view that sialylation is probably less than 50 percent complete as transfer occurs even on the native substrate. Ronzio has reported little galactosyltransferase activity in the presence of bovine mucin without prior hydrolysis by sialidase or by acid (121). In contrast, these data give native bovine mucin about 50 percent of the activity of acid treated material. Different batches of bovine mucin were examined by SDS-polyacrylamide gels and significant variability in protein staining bands was noted. Significant variability in the sialylation of mucin oligosaccharides may similarly exist between batches of commercially available material.

The basal activity with native fetuin can similarly be explained by microheterogeneity of the "complex type" oligosaccharide chains as well as transfer to the "mucin type" oligosaccharides (serine-threonine-linked) on fetuin.

An approximate doubling of galactose transferred was observed when the remaining NANA residues were cleaved from the mucin substrate by mild acid hydrolysis. The mucin activity was approximately twice that of fetuin on a per mg protein basis. The reasons for this could reflect on the relative activities of the transferase(s) to utilize N-acetylglucosamine versus N-acetylgalactosamine as substrates. Another explanation for the differences in activity is that bovine mucin has several fold the number of oligosaccharide chains as fetuin and the number of available acceptor sites is larger. Direct competition experiments with mixtures of mucin and fetuin in the assay system have not conclusively shown direct substrate competition between the N-acetylglucosamine of fetuin and the N-acetylgalactosamine of mucin. Protein substrates in all assays were present at concentrations less than saturating for enzymatic transfer, i. e. Figure 10. The assays were run at less than saturating conditions because of experimental difficulties encountered in the viscosity of the assay mixture when protein substrates were present at concentrations above 10 mg/ml.

The role of the protein acceptor in glycosyltransferases is extremely important. In some cases it may be postulated that the use of inappropriate acceptors is the cause of lack of correlation between phenomena, as in the lack of correlation between increased sialyltransferase activity and the transformed state of the cells (168). A

sialyltranferase from BHK transformed cells transferred sialic acid at a greater rate than its non-transformed parent line to a cellular glycopeptide fraction while transfer to non-specific acceptors (fetuin and mucin) was equal for both cell lines (56).

In general the DSG-fetuin pancreatic galactosyltransferase had properties that were similar to galactosyltransferases examined in other tissues (169,170,171) as well as the pancreatic mucin galactosyltransferase (121). The enzyme had an absolute requirement for Mn++ although as with the mucin transferase the activity could be partially restored with magnesium. Triton X-100 was required for activity with both enzymes.

In the pancreas neither the mucin nor the DSG-fetuin galactosyltransferase were affected by the addition of reducing agents to the reaction mixture.

Attempts were made to examine the endogenous reaction products by running the reaction without exogenously added protein acceptors. Incorporation of radioactivity into endogenous products was inhibited by Triton X-100, which suggests that after cellular disruption endogenous glycosylation is predominantly "cis". Endogenous incorporation was extremely low under all conditions examined. A study of endogenous pancreatic galactosyltransferase activity has been published by Ronzio and Mohrlok (120).

One of the reasons for characterization of this glycoprotein galactosyltransferase was the evidence pertaining to its involvement in the process of secretion. As the work of Paladi et al. (123) has shown, secretory proteins originate in the RER and consequently are transported to the smooth ER, then into the peripheral elements of the Golgi system where they bud off into specialized storage vesicles known as zymogen granules. The Golgi complex contains glycosyltransferases that add terminal sugars to core oligosaccharides of the "complex type". These terminal glycosyltransferases presumably glycosylate membrane glycoprotein constituents as well as secretory products. The distribution of the DSG-fetuin galactosyltransferase in pancreatic subcellular fractions supports its role as a terminal transferase (Table 3). Activity is enriched in the microsomal fraction as expected for a membrane-bound enzyme. Activity is less in the rough microsomal fraction than in the crude microsomes, leading to the conclusion that RER activity is primarily derived from contaminating smooth microsomes. The smooth microsomal fraction shows a 30 fold increase in activity over the crude homogenate and a 10 fold increase over the crude and rough microsomal fractions. The smooth microsomal fraction contains elements of the smooth endoplasmic reticulum, plasma membrane, and Golgi complex.

Glycosyltransferase activity on the plasma membrane has been the subject of dispute since Roseman first published his hypothesis on intercellular adhesion through cell surface glycosyltransferases (172). The glandular morphology of pancreatic acinar cells into duct systems probably excludes a major role of plasma membrane associated galactosyltransferase in this system for the following reasons. The cells are joined to one another by tight junctions and in tissue disruptions these membrane junctions do not purify into the smooth microsomal fraction. Futhermore the basal and apical cell membranes have relatively small surface areas in relationship to acinar cell size and would be diluted out by the large amounts of smooth endoplasmic reticulum present. It can thus be inferred that the DSG-fetuin galactosyltransferase located in the smooth microsomal fraction is primarily derived from the smooth endoplasmic reticulum and the Golgi vesicles. This conclusion is supported by the studies of Ronzio, who purified Golgi enriched fractions from rat pancreas and found over 50 percent of the total mucin galactosyltransferase localized there (121). The morphology of the Golgi enriched fraction was examined by electron microscopy to verify its composition and was found to contains stacks of cisternae associated with vesicles as well as smooth membranes of uncertain etiology. No contamination from RER or zymogen granules was found.

The zymogen granule fraction contains less than 0.1 percent of the total galactosyltransferase activity confirming the report of Ronzio that no mucin galactosyl-transferase activity was associated with the ZG membrane (121). The zymogen granule membrane has been purified and shown to contain a major glycoprotein species, located at the external face of the granule, with a molecular weight near 80,000 (119). Galactose is a major constituent of this glycoprotein and galactosylation of this component may be a signal in determining the preparatory state of the zymogen granule for secretion. Lack of transferase activity in the ZG fraction indicates that if galactosylation of ZG's is a point of regulatory control it is not cis-glycosylation. Zymogen granule membane glycoproteins may not be fully galactosylated upon formation (microheterogeneity of the oligosaccharides) and consequently their galactosylation might serve as a regulatory process. This was tested by assaying purified ZG membranes for acceptor properties. No acceptor activity was found when the purified membranes were run in the galactosyltransferase assay. It is unlikely that galactosylation of the ZG occurs after formation without invoking more complex processing reactions such as the removal and subsequent re-addition of galactose. This may occur, as terminal carbohydrate has been reported to turn over faster than the protein core (51).

Zymogen granule membrane precursors are probably glycosylated in part in the Golgi complex prior to their association into ZG's. Another possibility for regulation by glycosylation would involve glycosylation as a signal to initiate vesicle formation from the Golgi complex.

The possibility must be considered that although direct transfer of galactose from UDP-galactose to protein precipitable material appears to occur without a lipid intermediate (Figure 7) "in vitro", the "in situ" topographic locations of substrate, lipid intermediate, and enzyme may be such that direct transfer does not readily occur without a dolichol-type intermediate. The complex sub-cellular morphology present in the pancreatic acinar cell (Figure 3) certainly implies a high order of "in situ" organization. Exogenous dolichol phosphate was added to the assay system with smooth microsomes as the enzyme source and transfer to chloroform-methanol soluble material was examined. In no case was total galactosyltransferase activity increased nor was significant transfer to the lipid phase seen. Other investigators have reported an increase in galactose incorporation into protein in cell culture by the addition of dolichol phosphate (173).

In addition to galactose, zymogen granule membrane contains N-acetylglucosamine, fucose, and sialic acid. Other terminal transferases may be involved as well as galactosyltransferase in the regulation of secretion by glycosylation. Volkl et al. (174) has identified a fetuin

fucosyl transferase in the smooth microsomal fraction from the rat exocrine pancras. It has many similarities to the galactosyltransferase in subcellular distribution, activation by Triton X-100, and requirement for divalent catons (i. e. Mg or Mn). The possibilities that glycosyltransferases are associated in some unknown manner in the Golgi complex and their concerted action is necessary for complete terminal glycosylation of oligosaccharides in the Golgi complex is an attractive idea. Direct evidence for such a complex is presently not available.

Embryonic Rat Pancreas

The rat pancreas has served as a developmental model of differentiation and altered levels of galactosyltransferase activity are known to occur during pancreatic development (98). A DSG-fetuin galactosyltransferase was assayed as a function of embryonic age from day 14 to parturition (day 21-22). Its activity was found to parallel the levels found previously for a mucin galactosyltransferase by Carlson et al. (98) (Figure 8).

Several investigators have shown that high levels of pyrophosphatase activity can artificially lower apparent galactosyltransferase activity by hydrolyzing the UDP-[carbon 14]galactose substrate (97). To eliminate this effect on the galactosyltransferase activity, pyrophosphatase assays were set up and studies were conducted to determine an efficient inhibitor of

pyrophosphatase activity. Figure 14 demonstrates that in a galactosyltransferase assay with only labeled substrate, complete hydrolysis of UDP-Gal occurred after a 30 minute incubation. Boiling the mix before running the assay completely eliminated the hydrolysis(Figure 14).

Inhibitors of pyrophosphatase tested included: ATP,ADP,AMP,GMP, GDP, and CDP-choline. AMP was found to be the most effective inhibitor of pyrophosphatase activity tested (Table 4). AMP was then demonstrated to have no direct inhibitory or stimulatory effect on galactosyltransferase activity (Table 5). Nucleoside phosphates have been reported to have inhibitory and stimulatory effects on glycoprotein galactosyltransferases. In rat brain microsomes, GTP stimulated the transfer of [carbon 14]galactose from UDP-Gal to endogenous protein acceptors (175) while inhibiting transfer of [carbon 14]N-acetylglucosamine from UDP-GalNac (176). These dual effects argue against a role for GTP as simply an inhibitor of substrate hydrolysis by pyrophosphatase activity. Consequently, it was postulated that GTP acts as an allosteric modulator of glycosyltransferase activity (175).

When AMP (3.6mM) was included in the galactosyltransferase assay for embryonic pancreas no additional differences were seen in enzyme levels. Alterations in galactosyltransferase activity in embryonic rat pancreas are therefore not due to degradation of substrate by pyrophosphatase activity.

Neonatal Rat Pancreas

Galactosyltransferase assays were then extended to neonates. A small rise in activity had previously been seen 3-4 days postpartum. When these assays were run in the presence of 3.6 mM AMP a large induction of enzyme appeared at this point (Figure 19). This implied an induction of pyrophosphatase activity at the same point. Pyrophosphatase activity was then assayed directly (Table 6) and indeed a substantial increase in pyrophosphatase activity occured at postpartum day 3.

The large postnatal induction in galactosyltransferase activity was further examined. The activity was predominately microsomal (Figure 20) with no transition to a soluble form occurring as has been reported in developing chicken brain (103). Isoelectric focusing, polyacrylamide gel electrophoresis, and G-150 column chromatography revealed no differences between the adult and the neonatal enzymes. Other molecular parameters examined, pH optima, metal ion requirements, heat stability, and effect of reducing agents similarly revealed no differences. Mixing experiments failed to demonstrate the presence of stimulatory factors in the neonatal period.

The biological reason for the observed rise in galactosyltransferase activity in the neonatal rat pancreas is unknown. Several reasons for the alterations can be postulated. The development of the rat pancreas is probably

partially regulated by steroid hormones. Evidence has been published which implies a role for glucocorticoids in pancreatic development (177). A liver sialyltransferase is elevated by hydrocortisone (178) and a galactosyltransferase of the rat endometrium is enhanced by 17-beta-estradiol, diethylstilbesterol, estriol, and estrone (179). Consequently postnatal rises in hormonal activities may well induce transient stimulation of glycosyltransferase activity which then falls back to steady-state levels.

Galactosyltransferase Activity in Cultured Cell Systems

Cells grown in culture are an easily manipulatable system in which to study biochemical phenomena. The -DSG-glycoprotein: galactosyltransferase characterized in the pancreas was assayed in several cell lines and found to have similar if not identical requirements as the pancreatic enzyme.

Different chemical agents were added to cells in culture to determine their effect on galactosyltransferase activity. Some chemical agents may affect cells in such a manner that the cell becomes blocked in a specific stage of the cell cycle. If the galactosyltransferase activity changes throughout the cell cycle, as has been reported by some investigators (163), alterations in activity would be primarily a cell cycle effect. KB cells were synchronized by means of a double thymidine block and galactosyltrans-

ferase activity was assayed at two hour intervals thoughout one cell cycle (24 hours). No major alterations in galactosyltransferase activity were found (Figure 21). Consequently, any alterations induced in KB cells were not due to cell cycle blockage.

Nil-8 and Nil-8HSV Cells

Phorbol ester tumor promoters cause a variety of biochemical alterations in cultured cells (reviewed in 180). TPA, a potent tumor promoter, was added to Nil-8 and Nil-8HSV cells in culture. Results on galactosyltransferase activity are shown in Figures 24 and 25. TPA did not alter galactosyltransferase activity in Nil-8 cells but elevated it in Nil-8HSV cells. In an analogous fashion the anti-promoter, retinoic acid, had no effect on activity in Nil-8 cells but elevated activity in the Nil-8HSV cell line. When the promoter and anti-promoter were added together no synergistic effect was seen for either the Nil-8 or the Nil-8HSV cell lines. Alterations in galactosyltransferase activity are clearly more responsive in the transformed (Nil-8HSV) cell line than in the non-transformed counterpart (Nil-8).

CHO and CHO-M Cells

Alterations in galactosyltransferase activity have been observed when cells are preincubated with lectins. In the rabbit erythrocyte decreased galactosyltransferase activity results when the cells are preincubated with nonagglutinating concentrations of Con A (181). In BHK cells Weiser et al. (72) reported a correlation between Con A agglutination and galactosyltransferase activity and postulated that the existence of galactosyltransferase on the cell surface may be a requirement for Con A agglutination. However, subsequent studies related agglutination not to enzymic activity but to the presence of terminal alpha-linked mannose residues on the galactosyltransferase (182). When wheat germ agglutin (WGA) was bound to isolated rat liver Golgi membranes an increase in galactosyltransferase activity resulted (183).

CHO and a mutant cell line resistant to the cytotoxicity effects of wheat germ agglutin (CHO-M) were assayed for galactosyltransferase activity in the presence of TPA and retinoate in a manner identical to that for the Nil-8 cells. The CHO cells showed little induction of enzyme activity with TPA whereas the CHO-M cells showed a three-fold increase in activity. With retinoate the CHO cells showed a increase of activity of around 50 percent whereas the CHO-M cells showed an approximate doubling of activity.

Analogies between the Nil-8 and CHO cell systems can be drawn. The CHO-M cells react, as far as the induction of galactosyltransferase by TPA and retinoate, more like the transformed cell line Nil-8HSV than the untransformed line (Nil-8) which compares to the parental cell line CHO. Transformed cells generally have a less complex surface glycopeptide pattern than their untransformed counterparts (56). The exact nature of the molecular alteration in the CHO-M cells is not known but lessened lectin toxicity may imply defective surface carbohydrate structures. This may imply simpler cell surface glycopeptides analogous to transformed cells. An alternative explanation could be a mutation leading to the masking of the lectin recognizable carbohydrates. In at least one case lectin resistant cells have been shown to lack a specific glycosyltransferase (184).

Since a difference in galactosyltransferase activity was seen between CHO and CHO-M cells to the phorbol ester tumor promoter TPA, it was decided to test a graded series of phorbol ester tumor promoters to determine if a correlation could be found between the "in vivo" promoting activity and the induction of galactosyltransferase activity in either cell line. Results are shown in Figure 29. CHO cells showed a small response to all promoters tested with depressions as well as elevations of activity being seen. CHO-M cells showed elevation of activity for all promoters tested with a large elevation in activity observed for PDD

(100 percent) and PMML (40 percent). A clear correlation between "in vivo" promoting activity and galactosyltransferase activity was not observed. However, a higher elevation of activity did occur with the stronger promoters (PDD and PMML).

Endogenous Phosphorylation Studies in CHO andCHO-M Cells

In an attempt to elucidate the mechanism of galactosyltransferase alterations by tumor promoters, plasma membrane fractions from CHO and CHO-M cells were purified according to the method of Thom et al. (157). These membrane preparations were assayed for galactosyltransferase activity "in vitro" in the presence of phorbol ester tumor promoters. No induction of activity for any promoter was seen in membrane preparations of either CHO or CHO-M cells.

Interactions at cell membranes frequently involve the phosphorylation or dephosphorylation of proteins. A recent speculation was that a cyclic AMP protein kinase might be involved in the regulation of glycosyltransferases (185). Consequently a more primary event in cell surface tumor promoter interactions may be phosphorylation followed by alterations in glycosyltransferase activity. These secondary alterations would be disrupted by the purification of the plasma membrane fraction and would account for the lack of direct stimulation of galactosyltransferase activity by promoters "in vitro".

For neither CHO nor CHO-M cells, as for galactosyltransferase activity, is there an apparent correlation between "in vivo" promoting activity and phosphorylation.

These data do not strongly support a direct correlation between either phosphorylation or DSG-fetuin galactosyltransferase activity and "in vivo" promoter activity in either CHO or CHO-M cells. However, certain correlations can be noticed. The strongest "in vivo" promoters assayed (PDB, PDD, and PMML) altered both galactosyltransferase (elevation) and phosphorylation (depression) in CHO-M cells. The CHO cells had a much smaller response to the promoters, both in regard to galactosyltransferase and to endogenous phosphorylation.

When CHO and CHO-M cells were exposed to butyrate, effects were seen on galactosyltransferase activity (Figure 28) and on phosphorylation (Figure 36). Butyrate elevated galactosyltransferase activity in CHO and CHO-M cells. Effects on phosphorylation, however, are not uniform. Butyrate approximately doubles phosphorylation in CHO cells while having little effect on CHO-M cells (Figure 36).

Butrate has been reported to be an anti-promoter, and the following correlations can be summarized for the CHO and CHO-M cell systems: The strong promoters induce galactosyltransferase in CHO-M cells but not in CHO cells. Their effect on phosphorylation is a depression for CHO-M cells, but no effect on CHO cells. Butyrate induces galactosyltransferase in both CHO and, to a lesser extent, CHO-M

cells, but has no effect on phosphorylation in CHO-M cells while inducing phosphorylation in CHO cells.

KB Cells

Galactosyltransferase activity in human epithelial carcinoma (KB) cells was elevated in response to TPA and retinoic acid (Figure 26), and a significant negative synergistic effect was seen when retinoic acid and TPA were combined. No such effect was noted with either Nil-8 or Nil-8HSV cells. Epidermal growth factor (EGF) may have altered binding sites in the presence of TPA (186,187), and EGF did negate the induction of galactosyltransferase activity by TPA. A positive synergistic effect on galactosyltransferase activity was noted when EGF plus retinoic acid was assayed. Retinoic acid has been reported to be an anti-proliferation substance and it demonstrates opposing synergistic effects with TPA and EGF.

Autoradiography

Autoradiograms of endogenous phosphorylation of CHO and CHO-M cells exposed to tumor promoters and anti-promoters are shown in Figures 38 and 39. Clearly, differences in phosphorylation are seen between CHO and CHO-M cells. Cells grown in the presence of promoter and cell homogenages exposed to promoters "in vitro" show different effects. The anti-promoter butyrate greatly stimulates overall phosphorylation even when added "in vitro" to the assay systems.

Consequently, very different modes of effect may be inferred from the galactosyltransferase modification by butyrate than by tumor promoters.

Cell Surface Localization of Galactosyltransferase

The ability of cell homogenates to transfer galactose from UDG-Gal to monosaccharides was tested utilizing glucose and N-acetylglucosamine as acceptors. Results for KB cells are seen in Table 7. Transfer did occur and the modifier protein alpha-lactalbumin successfully modified acceptor specificity from N-acetylglucosamine to glucose as in the soluble galactosyltransferases purified from milk (reviewed in '88). This similarity in alpha-lactalbumin binding to the membrane-bound cellular enzyme allows the assumption that certain binding regions on both the soluble milk enzyme and the membrane bound enzyme are similar. These similar regions could allow for antibody cross-reactivity between antibodies raised against the easily purified milk enzyme and the difficult-to-purify membrane-bound enzyme.

Consequently, purified galactosyltransferase (milk enzyme) was obtained from R. Barker. This enzyme preparation yielded a single band on SDS-polyacrylamide gel electrophoresis. After concentration, 10 ug of purified enzyme was injected into New Zealand White Rabbits, and after a secondary booster injection, blood was collected and the IgG fraction was purified from the plasma. This IgG

fraction yielded a single immunodiffusion band against concentrated antigen (purified galactosyltransferase), while a pre-immune sample was negative (Ouchterlony immunodiffusion). Further characterization of the antibody was not performed.

Using double antibody immunofluorescence, CHO and CHO-M cells were examined for surface antibody binding. Experiments have shown cell surface associated immunofluorescence in the CHO-M cells but not in the CHO cells. In addition, purified plasma membranes from CHO-M cells had a higher DSG-fetuin galactosyltransferase activity than membranes from CHO cells (data not shown). A possible difference in plasma membrane associated galactosyltransferase has thus been implied between CHO and CHO-M cells. As mentioned previously, Con A binding has been directly associated with cell surface galactosyltransferases. These data show a reverse phenomenon, namely, cell surface galactosyltransferases correlated with lectin (WGA) resistance.

It should be kept in mind that the presence of cell surface glycosyltransferase may be of no functional significance. If cell membrane is derived from the Golgi apparatus (109) one would expect a plasma cell membrane with characteristics (i. e. glycosyltransferases) of the internal cell membrane from which it is derived.

Conclusions

A DSG-fetuin glycoprotein galactosyltransferase has been found to vary in activity during pancreatic differentiation. Other alterations in galactosyltransferase activity were found in cultured cell systems exposed to tumor promoters, anti-promoters, and other chemical agents.

Phosphorylation and galactosyltransferase activity may be modified in concert as a general membrane regulatory process. A direct correlation has not been established between a glycoprotein galactosyltransferase and endogenous phosphorylation, but correlation in cells exposed to tumor promoters and anti-promoters indicate a possible link.

Prospects

By studying alterations in a glycosyltransferase, we are attempting to gain information on how regulation of glycoconjugates effect cellular processes. Alterations in glycoconjugates are clearly important in many biological phonomena; however, they are not usually life-threatening. A mutant cell line, Balb 3T3B, which lacks the enzyme that acetylates D-glucosamine-6-phosphate, has been described (189). Drastic alterations are found in the oligosaccharides of its glycoproteins and extensive alterations in morphology and adhesiveness to substration are found but the cells survive.

Carbohydrate moieties may not be determinants of critical processes upon which the cell depends for survival, but act as modulators of processes which are the basis for intercellular social interactions (56). Transformation leads to alterations in the oligosaccharides of all subcellular membranes (nuclear, mitochondria, and endoplasmic reticulum) (190,191) but clearly transformed cells are viable. If alterations in oligosaccharides exceed tolerable limits the cells would not survive and such alterations are not approachable experimently.

A fundamental change in malignancy may be in genes concerned with membrane synthesis. Consequently, regulating agents in the membranes would be modified, which could be the glycosyltransferases.

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