STUDIES ON THE REGULATION OF GLYCOSPHINGOLIPID BIOSYNTHESIS

BY

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A DISSERTATION

Submitted to
Michigan State Unversity
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1984

ABSTRACT

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Three phorbol esters of varying tumor-promoting activity were used to probe regulation of glycosphingolipid biosynthesis. Treatment of cultured cells with phorbol esters resulted in increased incorporation of [1-^{14}C]palmitate and [9-^{3}H]sialic acid into gangliosides, and in accumulation of gangliosides.

CMP-sialic acid:lactosylceramide sialyltransferase was partially characterized. The apparent K_m values for CMP-sialic acid and lactosylceramide were 0.16 mM and 0.11 mM, respectively. The enzyme required Mn^{+2} for maximal activity, while Mg^{+2} and Ca^{+2} activated the enzyme to a lesser extent. Triton CF-54 (0.3 percent) compared to other nonionic detergents gave the greatest enzyme activation, while ionic detergents inhibited the enzyme. A broad pH optimum (4.5-8.0) was obtained, with maximum activity at pH 6.5. Sialyltransferase activity was highest in the M/early G_1 and G_1 phases of the cell cycle and in the contact-inhibited phase of cell growth. Phorbol esters increased the activity of the sialyltransferase.

The sialyltransferase was enriched by preparation of Golgi membranes, and solubilized from the membranes with a solution of 0.06

percent Triton X-100 and 40 percent glycerol. The enzyme was further purified by cellulose-phosphate and Sephadex G-150 chromatographic columns. Net purification was 127-fold with a recovery of 56 percent.

Phosphatidylcholine addition to the sialyltransferase resulted in approximately a ten-fold increase in enzyme activity, while phosphatidylethanolamine enhanced activity to a lesser extent and phosphatidic acid had no effect. Phosphatidylserine, phosphatidylinositol, lysophosphatidylcholine and lysophosphatidylethanolamine inactivated the enzyme.

Treatment of the sialyltransferase with bovine intestinal alkaline phosphatase resulted in reduction of enzyme activity. This reduction was dependent upon the amount of phosphatase used and length of treatment time. Phosphate prevented the reduction in enzyme activity while fluoride did not prevent the reduction in enzyme activity. Wheat germ acid phosphatase and bacterial alkaline phosphatase had no effect on activity. Pretreatment of the sialyltransferase with protein kinase increased the activity of the sialyltransferase, an effect which could be prevented by omission of phosphorylating agents or by the addition of protein kinase inhibitor. These results suggest the sialyltransferase may be regulated by phosphorylation-dephosphorylation.

ACKNOWLEDGMENTS

I would foremost like to thank my research advisor, Dr. Charles C. Sweeley, for his support and encouragement during my graduate research. His enthusiasm and guidance are appreciated.

I thank my co-workers in Dr. Sweeley's laboratory. Frank Wilkinson was a constant and bright source of help and friendship throughout everything. Barb Myszkiewicz provided many insightful discussions and was a true friend. Richard Lynch and Rob Soltysiak made my stay in the laboratory not only intellectually rewarding, but also fun.

Particular thanks go to my long time roommates Paul Bates and Dan Robinson. Both in their unique style invariably managed to make life in East Lansing interesting.

Special thanks to my good friends: Carol Fiol, Bob Swift, Maria Suarez, Arlyn Garcia-Perez and Theresa Fillwock.

Many thanks to Teresa Vollmer for the hard work and patience necessary for the typing of this dissertation.

John Burczak

July 17, 1984

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ABBREVIATIONS

N-acetylneuraminic acid is NeuAc or sialic acid; N-acetylgalactosamine is GalNAc; galactose is Gal; glucose is Glc; cytidine-5'-monophosphate is CMP; glycosphingolipid is GSL; glucosylceramide is GlcCer; lactosylceramide is LacCer; globotriaosylceramide is Gb3; globotetraosylceramide is Gb4; globopentaosylceramide is Gb5; II^3-α-N-acetyneuraminyl-lactosylceramide is G_{M3}; II^3-α-N-acetylneuraminyl- gangliotriaosylceramide is G_{M2}; 4α-phorbol-12,13-didecanoate is 4α-PDD; phorbol-12,13-dibutyrate is PDB; phorbol-12-myristate-13-acetate is PMA; phosphatidylcholine is PC; phosphatidylethanolamine is PE; phosphatidylinositol is PI; phosphatidylserine is PS; phosphatidic acid is PA; phosphate-buffered saline is PBS; NaAc is sodium acetate; Mes is 2-(N-morpholino)ethanesulfonic acid; Tris is tris-(hydroxymethyl)aminomethane; Hepes is 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Dulbecco's modified Eagle's medium is DME; fetal calf serum is FCS; fatty acids are designated as carbons:double bonds.

INTRODUCTION

I. Glycosphingolipid Biosynthesis.

Glycosphingolipids (GSL) are plasma membrane molecules composed of three components: a long-chain aliphatic amine, a fatty acid moiety, and a carbohydrate moiety [1]. The long-chain aliphatic amine has been found to be sphingosine (4-sphingenine) or a derivative thereof. The sphingosine is linked to a fatty acid via an amide linkage; the fatty acid can be either saturated or unsaturated and varies in length from 14 to 26 carbon units. Together the sphingosine and fatty acid components constitute the hydrophobic ceramide portion of the molecule, which is anchored in the membrane. The hydrophilic carbohydrate portion, varying in size from a monosaccharide to a complex oligosaccharide of 30 to 60 units, is attached to the ceramide C-1 hydroxyl group by a glycosidic linkage [1]. GSL which contain sialic acid in their carbohydrate portion are referred to as gangliosides [1].

The biosynthesis of sphingosine is believed to be initiated by the reaction of palmitoyl CoA with L-serine, followed by reduction and oxidation reactions to form 4-sphingenine or a derivative [2,3]. The biosynthesis of GSL from sphingosine occurs by two alternative pathways. The major pathway probably involves acylation of the sphingosine, followed by the transfer of a monosaccharide from a sugar nucleotide to the ceramide moiety [4,5]. A minor pathway may involve the transfer of galactose or glucose from a sugar nucleotide to

sphingosine, followed by acylation [6,7]. The oligosaccharide portion of the GSL is believed to be synthesized by the sequential addition of monosaccharides to the non-reducing end [8].

Based on many different precursor-product studies the pathways for neutral GSL and ganglioside biosynthesis were determined, Figures 1 to 3. The synthesis of glucosylceramide (GlcCer) and galactosylceramide (GalCer)from ceramide was reported by several groups using *in vitro* assay techniques [9]. Basu *et al.* [10] demonstrated the synthesis of lactosylceramide (LacCer) from GlcCer in embryonic chicken brain, while Hildebrand and Hauser [11] demonstrated the synthesis of LacCer and trihexosylceramide (Gb3) from GlcCer and LacCer, respectively in rat brain. Globoside (Gb4) synthesis from Gb3 was also characterized in embryonic chicken brain, and Forssman GSL (Gb5) synthesis from Gb4 was reported in guinea pig [12,13]. Thus, the precursor-product relationship for the synthesis of Gb5 in addition to a blood group B-active GLS [14] defined two pathways for neutral GSL biosynthesis, called the *globo* and the *lacto* series, respectively. The glycosyltransferases involved in these pathways have all been partially characterized, and are believed to be located on the lumenal side of the Golgi apparatus.

The gangliosides may be classified as acidic GSL containing N-glycolyl- or N-acetylneuraminic acid moieties attached to two distinct families of oligosaccharide core structures, the *ganglio* core (Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer) and the *neolacto* core (Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer). The biosynthetic pathways for the *ganglio* series of gangliosides were identified by Basu *et al.* [15], and have since been supported by several groups working with frog brain [16], rat brain [16], and neuroblastoma cells [17]. The biosynthesis

Figure 1. General biosynthetic pathways for glycosphingolipids.

GLYCOSPHINGOLIPID BIOSYNTHETIC PATHWAYS

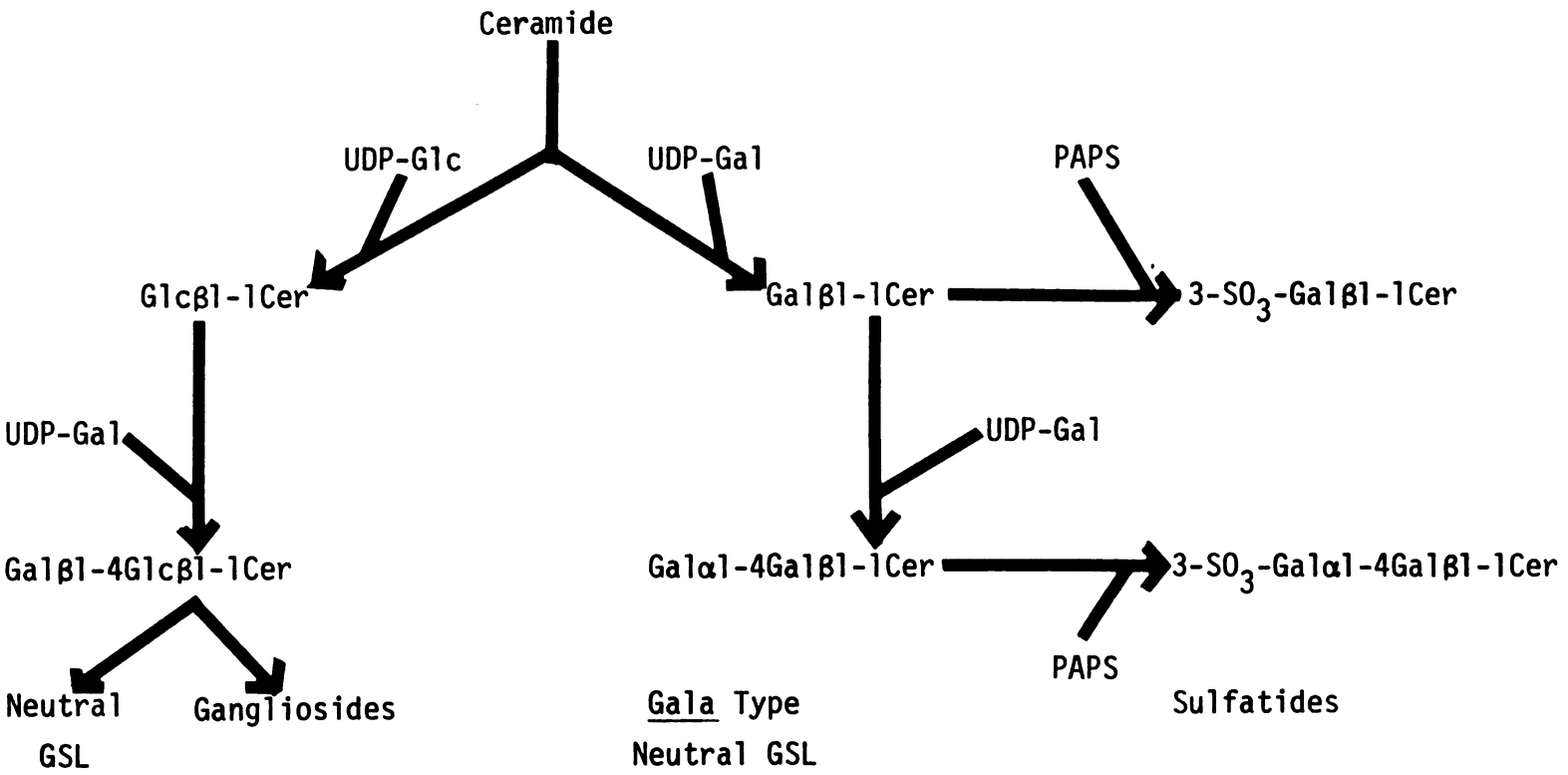


Figure 2. Biosynthetic pathways for gangliosides from lactosylceramide. Dashed line is an alternative pathway which has been proposed.

GANGLIO TYPE GANGLIOSIDE BIOSYNTHETIC PATHWAYS

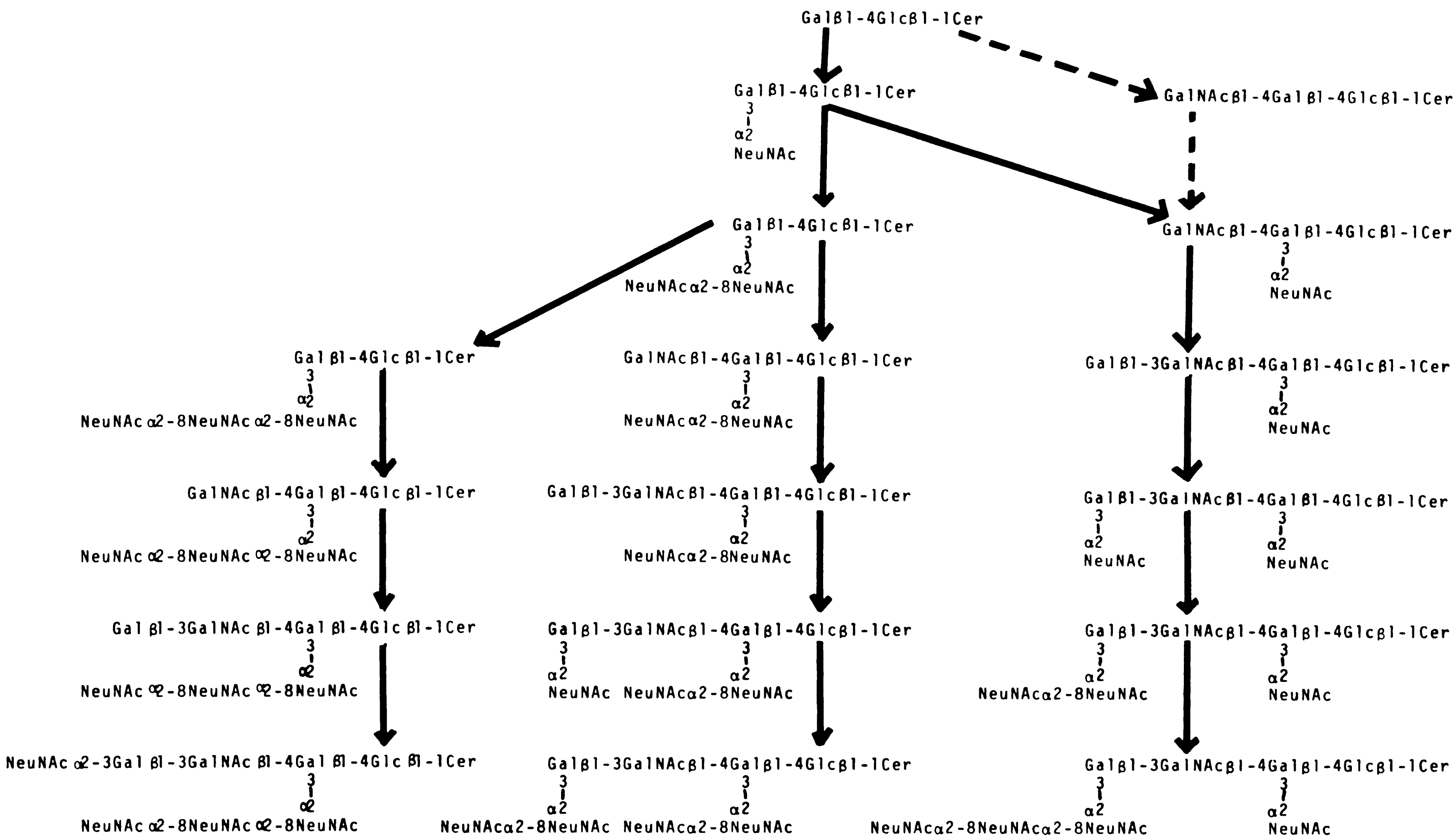
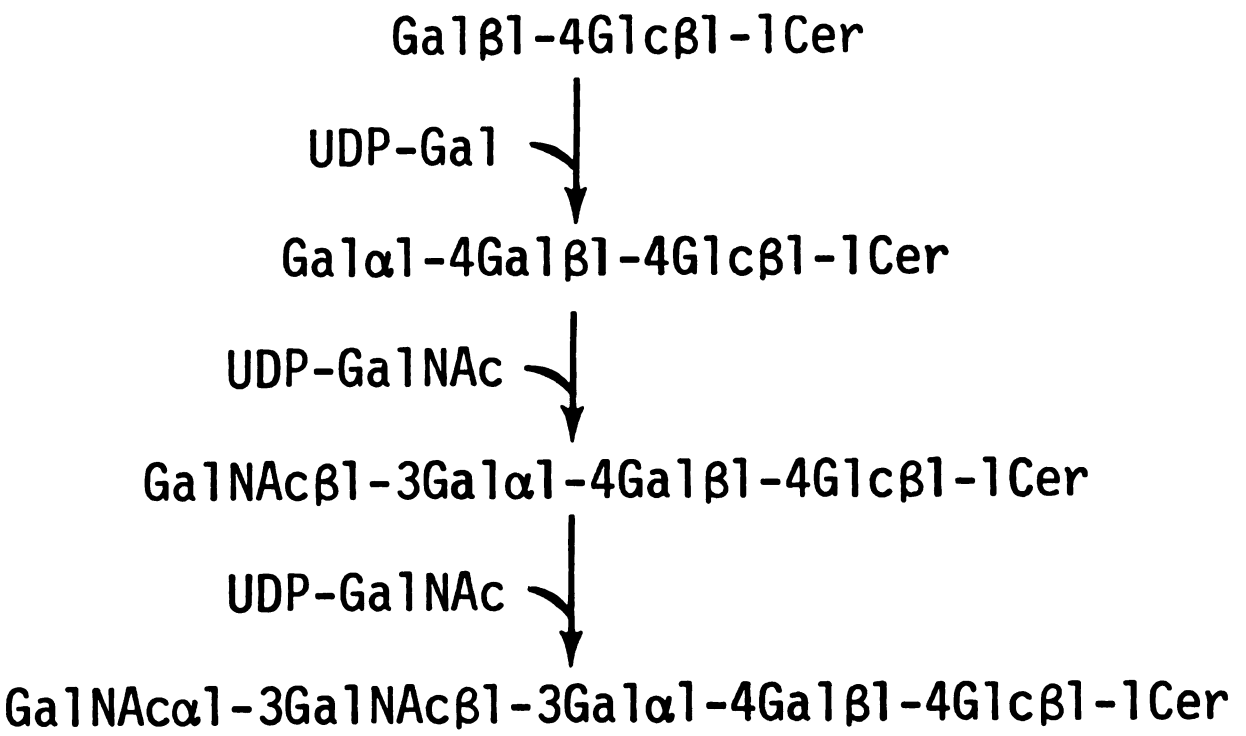


Figure 3. Biosynthetic pathways for Globo type neutral glycosphingolipids.

GLOBO TYPE NEUTRAL GLYCOSPHINGOLIPIDS



of the lacto series of gangliosides has not been determined in detail; however, precursor-product studies have revealed initial reactions in the pathways [18]. Recent kinetic data has revealed that the galactosyltransferases assayed during the in vitro synthesis of both LacCer from glucosylceramide and ganglioside G_{M1} from G_{M2} are independent catalytic entities [19]. At present it is unknown if all steps in the GSL biosynthetic pathways are catalyzed by different enzymes. Other studies indicate perhaps not [20].

Lactosylceramide, formed by the transfer of galactose from UDP-galactose to GlcCer, is a key compound in the biosynthesis of other GSL. This compound is not only the precursor for the globo family of GSL, but also stands at the branch point of two possible alternative pathways for ganglioside biosynthesis (Figures 1-3). CMP-sialic acid:lactosylceramide sialyltransferase, which catalyzes the formation of ganglioside G_{M3} from lactosylceramide (Figure 2), is of special interest and potential importance in regulation because it catalyzes the first committed step in the biosynthesis of nearly all gangliosides. Ganglioside G_{M3} is also of special interest because it is a branch point for two alternative pathways of ganglioside biosynthesis [21] (Figure 2).

The subcellular location of glycosyltransferases involved in GSL biosynthesis has been the subject of much controversy. Subcellular fractionation [21], autoradiography [22], and immunofluorescence studies [23] indicate that glycosylation generally occurs in the Golgi apparatus. Several other studies have indicated the existence of cell surface glycosyltransferases which perhaps function to a lesser extent in the biosynthesis of GSL [24].

Glycosphingolipids are sequentially catabolized by exoglycosylhydrolases. Evidence for this is provided by the various glycosphingolipidoses which result in lysosomal storage of specific GSL [25]. These genetic disorders are the result of specific defective glycosylhydrolases. Since glycosylhydrolases have acidic pH optima and the glycosphingolipidoses are lysosomal storage disorders, it is assumed that GSL are catabolized in lysosomes [26]. In addition, GSL catabolism has been demonstrated with intact lysosomal preparations [27]. Water-soluble glycosidases such as hexosaminidases are active on water-soluble substrates, but have hardly any effect on their GSL substrates. In vitro, this difficulty can be overcome by the addition of suitable detergents [28]. In human tissues, proteins have been identified that facilitate the interaction between water-soluble enzymes and their lipid substrates [29]. The physiological importance of these "activator" proteins is demonstrated by the finding that certain GSL accumulation diseases occur due to a dificiency of the activator protein [30]. The activator protein for hexosaminidase A has been demonstrated to extract GSL monomers from micelles or liposomes and form water-soluble complexes with a stoichiometry of 1 mole of GSL per mole of activator protein. In the absence of hexosaminidase A the activator acts in vitro as a GSL transfer protein, transporting GSL from donor to acceptor membranes [31]. The hexosaminidase A activator protein is rather specific for ganglioside G_{M2}. Other GSL form less stable complexes with the activator protein and generally are between membranes transferred at a slower rate than ganglioside G_{M2} [31].

II. Regulation of Glycosphingolipid Biosynthesis.

Although the biosynthetic pathways for GSL have been well characterized, relatively little is known about their regulation. Dawson et al. [32] have proposed that phosphorylation-dephosphorylation may be involved in the regulation of a glycosyltransferase in the ganglioside biosynthetic pathway [33], one which catalyzes the transfer of N-acetylgalactosamine (GalNAc) to ganglioside G_{M3}. Ganglioside G_{M3}, the product of CMP-sialic acid:lactosylceramide sialyltransferase, is a key ganglioside in that it may be the substrate for the GalNAc-transferase or a second sialyltransferase, which can therefore be considered to initiate separate ganglioside biosynthetic pathways. The GalNAc-transferase activity in cells treated with prostaglandin E1 (PGE1), cholera toxin, 8-bromo-cAMP, dibutyryl-cAMP or phosphodiesterase inhibitors was found to be increased [32,33]. These are conditions that would inhibit the activity of cAMP dependent protein kinase in vivo. When enkephalins were added to these cells to block PGE1 or cholera toxin stimulation of adenyl cyclase, induction of GalNAc-transferase was inhibited.

Another level at which GSL biosynthesis may be regulated is at the availability of sugar nucleotides. Individual monosaccharides are added from sugar nucleotides to GSL to form a carbohydrate chain on the GSL molecule [8]. Rats injected with galactosamine were found to have a reduced in vivo liver biosynthesis of gangliosides G_{M1} and G_{D1a} [34]. This amino sugar leads to a change in hepatic

UDP-sugar levels with a lowered content of UDP-Glc and UDP-Gal along with an elevated content of UDP-hexosamines and UDP-N-acetylhexosamines. Ganglioside G_{M1} biosynthesis from G_{M2} involves the addition of galactose from UDP-Gal [35]. Ganglioside biosynthesis may therefore be impaired by the lack of UDP-Gal or by an inhibition of some galactosyltransferase due to increased levels of UDP-hexosamines and UDP-N-acetylhexosamines. Other studies have shown that CMP-sialic acid can penetrate microsomes in a manner suggesting carrier mediated transport [36]. Upon subfractionation of rat liver, both the highest CMP-sialic acid specific transport activity and total transport activity were localized in the Golgi apparatus. Any transport activity in the smooth and rough endoplasmic reticulum could be accounted for by contamination with Golgi membranes, as determined by Golgi membrane marker enzyme activities. Transport of CMP-sialic acid was temperature dependent, inhibited by pronase as well as substrate analogues, and saturable [37]. Such a transport mechanism could regulate the availability of sugar nucleotides for GSL biosynthesis. Tunicamycin has been found to inhibit ganglioside biosynthesis in neuronal cells [38]. The mechanism of inhibition is believed to involve the blocking of sugar nucleotide transport across the Golgi membrane [39,40].

III. Glycosphingolipid Function.

Glycosphingolipids are believed to be involved in a variety of cell surface phenomena [41-49]. GSL function has been classified into three groups according to their behavior [50]. The first, annular GSL surround a membrane protein, and form a complex with it [51]. By way

of example, 3'-sulfo-galactosylceramide is believed to complex with Na^+K^+ ATPase and serve as a K^+-selective cofactor [52].

The second type of GSL function is as a cell surface marker, such as GSL with blood group determinants [53], or as a cell surface receptor [44]. GSL, particularly gangliosides, have been demonstrated to interact with various biologically active factors [54] (Table 1). Inhibition or modulation by GSL of the physiological effects of these factors on cells has been interpreted as showing that GSL function as receptors. This topic, particularly the subject of gangliosides as receptors for cholera toxin and glycoprotein hormones, has been extensively reviewed [55]. Although the majority of cell gangliosides are believed to be associated with the outer leaflet of the plasma membrane, certain amounts are found in the blood serum. It has been speculated that serum gangliosides released by tumor cells participate in protection from host immune rejection [56]. Recently [57], it has been demonstrated with mouse ascites hepatoma (MAH) cells, that the amount of gangliosides shed depends on the cell density. MAH cells incubated at 10^6 cells/ml were found to release about three times higher amounts of gangliosides per cell than during incubations at 10^8 cells/ml. The possibility was raised that the more intense shedding of gangliosides from less crowded MAH cells may play a role in the protection of the tumor from host immune rejection during initial stages of growth. The ganglioside composition of ascites fluid and conditioned cultured medium from MAH cells was found to be qualitatively identical to that of the MAH cells, but quantitatively different. The most polar cell ganglioside, chromatographically corresponding to G_{T1b}, was about ten times higher in the extracellular fluids than

in the cells. The major portion of the released gangliosides were found to be associated with a 150,000xg pellet that probably contained shed plasma membrane fragments.

The third type of GSL function is membrane matrix formation [58, 59]. The ceramide moiety of GSL is assumed to be inserted into the outer leaflet of the lipid bilayer, and the carbohydrate moieties may extend into the outer environment of the cell [1]. Ceramide-containing compounds such as GSL have been demonstrated to confer greater membrane structural rigidity in liposomes than glycerides [60], perhaps because ceramide contains both a hydrogen acceptor (amide carbonyl group) and a donor (hydroxyl group) to form stable hydrogen bonds [61]. In contrast, glycerides have only a hydrogen acceptor (ester carbonyl group) [36,42]. Since GSL can form rigid, ordered membrane regions, these lipids may show specificity in regulating membrane conformation [61].

Glycosphingolipids are generally believed to function in these three modes while mediating cellular events such as growth control, differentiation and transformation [44].

IV. Glycosphingolipids and Growth Control.

Changes in GSL have been closely related to contact inhibition of cell growth, which suggests that GSL may be involved in the regulation of cell growth. Synthesis of the GSL Gb3 in BHK cells [63], Gb5 and G_{M3} in NIL cells [64,65], G_{D1a} in 3T3 cells [66], and G_{D3} and G_{M1} in human fibroblasts [45] are greatly enhanced prior to density-dependent growth inhibition. The enzymatic basis for many of these responses [13,67] and the loss of many of these responses have been correlated to a loss of density-dependent growth inhibition during oncogenic

Table 1. Suggested interaction of glycosphingolipids with bioactive factors [62].

Biofactor	Glycosphingolipid
Bacterial toxins	
cholera toxin	G_{M1}
tetanustoxin	G_{T1b}, G_{Q1b}
Botulinus toxin	G_{T1b}
Staphylococcus α toxin	sialylparagloboside
Gonococcus pilli protein	G_{M1}
Glycoprotein hormones	
thyrotropin	G_{D1b}
chorionic gonadotropin	G_{T1}
leuteinizing hormone	G_{T1}
Sendai virus [68]	G_{T1a}, G_{T1}
Influenza virus [69]	G_{M1}
Interferon (type 1 only)	G_{M2}, G_{T1}
Serotonin	G_{D3}
Migration inhibitory factor [70]	fucose containing ganglioside

transformation [63-66]. Chemically transformed mouse embryo cells show a GSL pattern similar to nontransformed cells but lack the density-dependent synthesis of G_{D1a} [71]. However, this lack of response may not be an absolute criterion for tumorigenicity, since there are exceptions [72].

To test the possibility that GSL may indeed play a role in cell proliferation, three approaches have been undertaken: incorporation of GSL into cell plasma membranes [73,74], addition of antibodies toward GSL to cultured cells, and measurement of GSL changes caused by cellular differentiation. Upon the addition of Gb4 to the plasma membrane of NIL cells, cell adhesiveness increases, G_1 phase of the cell cycle becomes twice as long, and the morphology of polyoma-infected NIL cells (NILpy) resembles that of normal cells [75]. Similarly, addition of a mixture of gangliosides to the membranes of normal and SV40-transformed 3T3 cells reduces both their growth rates and saturation densities [76]. Recently [77], it has been demonstrated that gangliosides G_{M3} and G_{M1} added to Swiss 3T3 cells resulted in inhibition of cell growth as determined by cell number. G_{M3} addition to the cells also inhibited both platelet-derived growth factor (PDGF) and epidermal growth factor stimulated mitogenesis, while G_{M1} could only inhibit PDGF stimulated mitogenesis, as determined by [^{3}H]thymidine incorporation. Both G_{M3} and G_{M1} inhibited PDGF stimulated tyrosine phosphorylation of a 170,000 molecular weight protein (presumed to be the PDGF receptors) by membrane preparations. Moreover, 3T3 cells whose growth was inhibited by G_{M3} or G_{M1} showed an increased affinity for PDGF as compared to cells without addition of gangliosides, while the total number of receptors stayed the same. No

direct interaction was seen between gangliosides and growth factors due to the lack of competition by ganglioside containing liposomes for cellular binding of [^{125}I] growth factors. Thus the levels of gangliosides G_{M3} and G_{M1} in membranes may modulate PDGF receptor function by affecting the degree of tyrosine phosphorylation and the affinity of the receptor.

Experiments both *in vivo* [78] and *in vitro* [79] have demonstrated that exogenously administered gangliosides penetrate into cells and are metabolized. Therefore, it cannot be excluded that some of the effects of GSL added to cell membranes are due to interactions of metabolized GSL.

The second approach to studying the function of specific GSL in the regulation of cell growth has been to observe changes following the addition of specific anti-GSL antibodies [80]. Anti-G_{M3}, but not anti-Gb4, markedly inhibits the growth of NIL cells, but not their transformed counterparts. Addition of anti-G_{M3} to subconfluent cultures leads to a stimulation of [^{14}C]galactose incorporation into GSL, particularly G_{M3}, whereas GSL synthesis in confluent cultures (where G_{M3} levels are already enhanced) is not stimulated. Thus, it has been postulated that the interaction with anti-G_{M3} mimics those cellular interactions that stimulate the density-dependent synthesis of G_{M3} and may lead to a loss of cell growth [80].

Sodium butyrate, a potent inducer of differentiation for various cell systems [81], when added to culture media reduces cell saturation densities and restores contact-inhibition of cultured cells [82]. Associated with these changes in cell growth behavior is a greatly increased level of G_{M3} due to an enhanced CMP-sialic

acid:lactosylceramide sialyltransferase activity. The tumor promoter PMA which induces differentiation of human melanoma cells, has also been found to induce the synthesis of melanin and ganglioside G_{M3} [83]. Various retinoid compounds added to culture media have caused transformed cells to regain their normal growth behavior and show density-dependent growth inhibition [84]. An increased synthesis of G_{M3} in NIL cells has been demonstrated after the administration of retinoic acid [85]. Thus, the mechanisms by which butyrate, phorbol esters and retinoids induce differentiation and changes in cell growth may in part be due to changes in membrane GSL.

V. Glycosphingolipids and Cellular Differentiation.

Embryonic or histogenic differentiation is the orderly process whereby cellular structures and functions of an organism progressively change, thereby giving rise to specialized cells and structures [86]. During differentiation, changes in GSL composition and synthesis have been found in several cells systems, indicating that GSL may function during this process [62]. Changes in cell surface molecules during embryonic development have been detected by immunological methods. In each case, the antigenic molecule was found to be a GSL or the carbohydrate moiety of a glycoprotein, and was expressed at defined stages of embryonic development [62]. A cell surface antigen, SSEA-1, detected by monoclonal antibodies, appears at the morula and blastocyte stage of embryonic development, then disappears upon further development [87]. The antigen has been identified as a GSL with a complex carbohydrate chain [88,89]. A teratoma is a tumor derived from embryonic tissue [86]. Under certain growth conditions a teratoma may be induced to

differentiate. The surface antigen TerC of mouse testicular teratoma 402AX is a GSL [90]. The GSL composition of teratocarcinoma cell lines is altered upon the induction of cell differentiation. The gangliosides G_{M1} and G_{D1a} appear upon teratoma cell differentiation [91].

The crypt cells of the intestinal epithelia progressively migrate to the top of intestinal villi within twelve hours. During this time the crypt cells undergo differentiation to villus cells and develop a well-defined brush border with digestive enzymes. The undifferentiated crypt cells are characterized by the presence of lactosylceramide and galactosylceramide and by the absence of ganglioside G_{M3} and CMP-sialic acid:lactosylceramide sialyltransferase activity. The villus cells are characterized by high levels of G_{M3} and CMP-sialic acid: lactosylceramide sialyltransferase activity and low levels of lactosylceramide and glucosylceramide. Ganglioside G_{M3} synthesis from lactosylceramide by sialyltransferase is therefore correlated with the rapid development of intestinal epithelia [92,93].

3T3-L1 cells, cloned from mouse embryo fibroblasts, differentiate in culture into cells exhibiting typical adipocyte morphology [94-96]. The differentiation of 3T3-L1 preadipocytes is accompanied by increased levels and activities of many enzymes [97-100], as well as alterations in cellular interactions with various hormones [101-103]. In general, these changes are consistent with the increased lipogenic capabilities of the cell. Differentiation of 3T3-L1 cells to cells having enzymatic and morphological characteristics of adipocytes is also accompanied by an approximately fifty percent decrease in total cellular ganglioside content. These losses were reflected primarily by changes in

ganglioside G_{M3} and G_{D1a}, which comprised greater than 95 percent of the total ganglioside content [104]. Large changes in total ganglioside content were not noted when nondifferentiating 3T3-C2 cells were analyzed under conditions similar to those for 3T3-L1 cells. Thus, the decrease in ganglioside content appears to be directly related to differentiation and probably does not result from the incubation conditions employed to induce differentiation.

Another system in which GSL appear to play a role is myoblast differentiation. The cytodifferentiation of nucleated myotubes in vitro has been found to occur via aggregation and fusion of mononucleated myoblasts, in both primary cultures [105] and established cell lines [106]. Cloned cells of the rat myoblast cell line L6 synthesize gangliosides G_{M3}, G_{M2}, G_{M1} and G_{D1a}. The amounts of G_{M3}, G_{M2} and G_{M1} do not vary significantly during the differentiation of myoblasts to myotubes. However, the concentration of G_{D1a} transiently increases almost three-fold just prior to fusion of myoblasts, and returns to basal levels in the myotubes [107]. Mutant myoblasts selected by 5-azacytidine resistance, synthesize only ganglioside G_{M3} and G_{M2} and were unable to fuse and differentiate [107]. Since differentiation of myoblasts to myotubes must involve alteration of membrane components, it may be concluded that ganglioside G_{D1a} participates in the process.

VI. Glycosphingolipids and Oncogenic Transformation

Many studies have been performed with cultured cells transformed by DNA tumor viruses, RNA tumor viruses and physical agents such as chemicals and radiation. GSL changes associated with oncogenic

transformation can be classified into seven types, Table 2 [44]. Types one to four represent incomplete GSL synthesis, while types five to seven represent the induction of new GSL types. Incomplete synthesis is believed to be due to an impairment or block in the normal enzymatic pathway of GSL biosynthesis. Such a block would eliminate or suppress the biosynthesis and expression of subsequent GSL. A density-dependent change in the GSL present in hamster BHK and NIL cells has been demonstrated by [^{14}C]palmitate labelling experiments [108]. Among the various GSL present, Gb3 content was enhanced in growth-inhibited cells. This GSL is synthesized by an α-galactosyltransferase from the key GSL, lactosylceramide. The activity of this α-galactosyltransferase is two- to three-fold greater in BHK and NIL cells when they are growth-inhibited than in cells at lower population densities [13]. The elevation of this transferase activity provides a molecular mechanism for the increased levels of Gb3. The same cells transformed by the tumorigenic polyoma virus, a DNA virus, displayed no change in Gb3 content at any cell density. The activity of the α-galactosyltransferase in the transformed NIL and BHK cells was 10 to 50 percent of that in normal growing cells, and was not influenced by cell population densities [13]. Therefore, Gb3 accumulation parallels density-dependent inhibition of cell growth. This process does not occur in transformed cells, presumably because either production of the needed transferase is decreased or the enzyme is inhibited.

RNA tumor virus transformation of Balb/c 3T3 cells results in a drastic alteration of cellular ganglioside composition [109]. The Kirsten murine sarcoma virus (KiMSV) transformed Balb/c 3T3 cell is unable to synthesize gangliosides G_{M1} and G_{D1a} due to a complete

Table 2. Types of glycosphingolipid changes associated with oncogenic transformation [62].

Type 1.	Decrease or deletion of G_{M3}, G_{D3}; LacCer or GlcCer increase.
Type 2.	Decrease at deletion of G_{T1}, $G_{D1a,b}$ increase of G_{M3}, G_{M2} or G_{M1}.
Type 3.	Accumulation of asialo core carbohydrates, which are normally absent.
Type 4.	Decrease or deletion of Gb3, Gb4, Gb5 or longer chain neutral glycosphingolipids.
Type 5.	Decrease or deletion of G_{M3}, G_{M2}; increase of G_D and G_T.
Type 6.	New synthesis of Gb5.
Type 7.	New synthesis of incompatible blood group antigen foreign to the host.

absence of a β-galactosyltransferase. In the ganglioside biosynthetic pathway, ganglioside G_{M1} is synthesized from G_{M2} via a β-galactosyltransferase, and ganglioside G_{D1a} is synthesized from G_{M1} via a sialyltransferase. Thus, absence of β-galactosyltransferase activity prevents the biosynthesis of both G_{M1} and G_{D1a}. The effect of transformation by KiMSV on GSL metabolism in these cells appears to be highly specific, in that there was reduction of no other glycosyltransferases in the ganglioside pathway [109]. A block in the biosynthesis of ganglioside G_{M1} also appears in other transformed cell systems as well. These systems include SV40-transformed human fibroblasts [63,109], spontaneously transformed mouse embryo cells [110], and x-ray transformed Balb/c 3T3 cells [109].

Hamster cells transformed by polyoma virus or treatment with the chemical carcinogen dimethylnitrosamine and revertants from these transformed cells have been examined for their ganglioside composition [111]. Compared to normal cells, both transformed and revertants had a simpler composition of gangliosides. Normal cells contained gangliosides G_{M3}, G_{M2}, G_{M1}, G_{D1a}, G_{D1b} and a trisialoganglioside. Both polyoma-transformed cells and their revertants contained G_{M3} and trace amounts of G_{M2}, and both dimethylnitrosamine-transformed cells and their revertants contained only G_{M3}. Both types of transformed cells and their revertants contained only three to twelve percent of the β-N-acetylgalactosaminyltransferase activity of normal cells, an activity needed for the synthesis of ganglioside G_{M2} from G_{M3}. The decrease in GalNAc transferase activity provides a molecular mechanism for the simplification of the ganglioside types synthesized by the transformed cells and their revertants. The

presence of a similar decrease in both glycosyltransferase activity and ganglioside biosynthesis in both the transformed cells and in the revertants indicates that this process may not be associated with the regulation of cell growth but with a common genetic change from normal in transformed cells and their revertants. Although these revertants had reverted in several biological properties characteristic of transformation, such as the suppression of the ability to form tumors in animals and a high growth saturation density, they were no longer diploid and their chromosome constitution differed from that of normal cells.

In addition to incomplete GSL biosynthesis, oncogenic transformation has also resulted in the biosynthesis of new GSL species containing one or another novel carbohydrate moiety [62,112]. GSL containing an A-like blood group antigen has appeared in tumors of blood group B or O individuals [101,113]; its structure in one case was determined to be a difucosylated heptasaccharide with an A blood group determinant [114]. The GSL Gb5 is absent from gastrointestinal mucosa of the majority (70 to 80 percent) of humans, whereas gastrointestinal tumors of these individuals often contain this GSL [115].

Human teratocarcinoma cell lines express a stage-specific embryonic antigen, SSEA-3, detected by a monclonal antibody raised against four- to eight-cell-stage mouse embryos [116]. The antigen is expressed in a stage-specific manner during early mouse embryogenesis and a change in the expression of SSEA-3 is also detected during the course of differentiation of human teratocarcinoma cells [117]. The presence of an embryonic antigen common to mouse embryos and human teratocarcinoma cell lines is of interest because of the possibility of

conserved expression of such an antigenic determinant on functionally related cells from different species. Recently, GSL purified from human teratocarcinoma cells have been found to be reactive with anti-SSEA-3 monoclonal antibodies [118]. The structures of these GSL have been found to represent a new series of GSL. There are many other examples of the biosynthesis of new GSL species during oncogenic transformation. The subject has recently been reviewed by Hakomori [62].

VII. Tumor Promotion and Glycosphingolipids.

One well-accepted theory of carcinogenesis is the two-stage theory, the basis of which is that tumors result from a combination of an initiation and a promotion event [119,120]. Most initiators or carcinogens are chemical or physical agents which directly damage DNA [121]. Tumor promoters or cocarcinogens are compounds which are not carcinogenic, but which cause the outgrowth of tumors when applied repeatedly after a single subthreshold dose of a carcinogen. Compounds capable of both initiation and promotion are termed complete carcinogens. The two-stage theory of carcinogenesis has been studied most thoroughly in mouse skin [122,123]. In mouse skin assays it was found that: (a) a small subthreshold dose of a carcinogen resulted in no tumor formation; (b) multiple applications of a tumor promoter resulted in no tumor formation ; (c) application of a tumor promoter followed by a subthreshold dose of a carcinogen resulted in no tumor formation; however, (d) a subthreshold dose of a carcinogen followed by repeated tumor promoter application resulted in tumor formation [123].

Moreover, if a tumor promoter was added one year after initiation tumors resulted, indicating that initiation may be permanent [124].

Esters of the tetracyclic diterpene, phorbol, are among the most potent tumor-promoters in standard mouse skin assays [125]. Their activity is highly dependent on the position and chain length of the ester substituents, as well as on the three dimensional conformation of the molecule [126]. The most active phorbol tumor promoter phorbol-12-myristate-13-acetate, PMA, is both inflammatory and tumor promoting [127]. Several phorbol derivatives have been identified which are highly inflammatory but either nonpromoting or only weakly promoting [128]. In conjunction with in vitro structure-activity studies [126], this result suggests that more than one cellular target may exist for the phorbols and structurally related diterpenes to elicit their biological effects. There is also a good correlation between the tumor promoting capacity of a series of phorbol compounds and their ability to stimulate epidermal hyperplasia [129]; however, the correlation fails with nonphorbol hyperplastic agents [130]. It is difficult to determine which of the many responses related to phorbol tumor promotion are essential components of the promotion process. The tumor promoting ability of various compounds (phorbol as well as nonphorbol) and their ability to induce ornithine decarboxylase activity in mouse skin has been demonstrated to correlate well [131]. However, mezerein, a diterpene similar to phorbol, is capable of bringing about most of the biochemical and morphological changes seen with PMA, especially the induction of cell proliferation and onithine decarboxylase activity, but PMA was at least fifty times more active as a promoter [132].

Recently, the promotion aspect of the two-stage theory of carcinogenesis has been divided into two phases due to results of experiments using various combinations and sequences of weak tumor promoting agents after initiation of mouse skin by a subthreshold dose of a carcinogen [133,134]. The first stage of promotion has been termed conversion and the second stage has been termed propagation. Although the nature of the conversion step is not understood, the propagation step is characterized by hyperplasia and an increase in ornithine decarboxylase activity [135]. Strong tumor-promoting compounds that act at both stages of promotion have been termed complete promoters, while weak tumor-promoting compounds that act at only one stage of the promotion process have been termed incomplete tumor-promoters. This may explain why the hyperplastic and inflammatory properties of tumor-promoters generally correlate with tumor-promotion, but with exceptions. Indeed, incomplete promoters seem to be the compounds that are the exceptions to the hyperplastic and inflammatory correlation with tumor promotion [134].

In addition to causing epidermal inflammation and hyperplasia, the phorbol ester tumor-promoters have been shown to have several other effects on mouse skin: they alter skin cell morphology [136], decrease epidermal differentiation [137], induce a new cell type called dark cells in adult skin [138], induce embryonic proteins in adult skin [139], increase RNA, protein and DNA synthesis [140], increase phospholipid synthesis [141], increase protease activity [142], increase ornithine decarboxylase activity [106], and increase histone synthesis and phosphorylation [143,144]. Furthermore, the phorbol esters have

been shown to have several effects on cells in culture. This area has been extensively reviewed by Blumberg [127].

Recent studies with JB6 cells indicate that GSL may be involved in tumor-promotion [145,146]. Anchorage-independent growth and tumorigenicity are inducible in JB6 mouse epidermal cells by tumor-promoting phorbol esters [147]. These pre-neoplastic cells are useful for studying *in vitro* promotion of transformation because they respond to phorbol esters and other promoters by irreversibly shifting to a tumor cell phenotype. Exposure of JB6 cells to PMA decreased *de novo* synthesis of trisialoganglioside to five to ten percent that of untreated cells [146]. In addition, the decrease in trisialoganglioside synthesis occurred in promotion-sensitive, but not promotion-resistant clonal variants of the JB6 cell line. Addition of trisialoganglioside into membranes of PMA-treated JB6 cells blocked the induction of anchorage independent transformation. This ability to inhibit the promotion of transformation was specific, in that other GSL such as G_{M1}, G_{D1a} and asialo-G_{M1} could not inhibit [146]. The mechanism by which trisialoganglioside blocked transformation appears to be after PMA binding to its receptors, since exogenously added trisialoganglioside did not inhibit specific binding of tritiated phorbol esters [146]. Direct oxidation of JB6 cell surface gangliosides by $NaIO_4$ led to both decreased ganglioside G_T synthesis as determined by radiolabeled precursor incorporation and promotion of anchorage independent transformation [148]. Promotion of transformation by $NaIO_4$ and PMA was specifically inhibited by the addition of ganglioside G_T. Neither oxidized G_T nor other gangliosides inhibited $NaIO_4$ promotion. Such a decrease of trisialoganglioside could result from

enzymatic or oxidative degradation. It was postulated that tumor-promoting free radical generators may have trisialoganglioside as a target in JB6 cells [148].

STATEMENT OF THE PROBLEM

The biosynthetic pathways of GSL have been well characterized; however, little is known about their regulation. The present study was designed to identify and characterize a regulatory site in GSL biosynthesis. The goals of this study were as follows:

a) Assessment of GSL changes in cultured cells due to treatment with tumor-promoting phorbol esters and determination of possible regulated enzymes in the GSL biosynthetic pathways.

b) Characterization of a possible regulated enzyme with respect to substrate binding, pH optimum, detergent requirements, metal ion requirements, activity at different stages of the cell cycle, and activity during cell growth.

c) Partial purification of the possible regulated enzyme in the GSL biosynthetic pathway.

d) Assessment of possible mechanisms by which this enzyme is regulated.

These studies will lead to a better understanding of the regulation of a specific glycosyltransferase, and enable future investigators to obtain more direct evidence for the role of GSL and their biosynthetic enzymes in cell growth regulation and transformation.

MATERIALS

Cell Culture Supplies

All plastic tissue culture flasks	Corning Glass Works Corning, NY
All types of growth media, fetal bovine serum and trypsin	GIBCO Laboratories Grand Island, NY

Chemicals

CMP-sialic acid, phorbol esters, phosphoenol-pyruvate, NAD^+, $NADP^+$, Tris, HEPES, MES, cacodylate	Sigmal Chemical Co. St. Louis, MO

Chromatography Supplies

Dowex-1 and cellulose-phosphate	Sigma Chemical Co. St. Louis, MO
Sephadex G-150 and Sephadex A-25	Pharmacia Fine Chemicals Inc. Piscataway, NJ
Silica Gel 60 High Performance Thin-Layer Chromatography Plates Iatrobeads	EM Reagents Darmstadt, Germany Iatron Laboratories, Inc., Japan

Detergents

sodium taurocholate (Grade A), sodium taurodeoxycholate (Grade A)	Calbiochem La Jolla, CA
Triton X-100, Triton CF-54, Tween 20, Tween 80, Cutscum and hexadecyltrimethyl-amonium bromide	Sigma Chemical Co. St. Louis, MO

Radio-labeled Materials

[6-^{3}H]N-acetylmannosamine, [methyl-^{3}H] thymidine, [1-^{14}C]palmitate and CMP-[4,5,6, 7,8,9-^{14}C]sialic acid	New England Nuclear Boston, MA

METHODS

I. Preparation of [9-^{3}H]Sialic Acid.

Synthesis of [9-^{3}H]sialic acid [149]. Ten grams of rat liver was homogenized in 20 ml of cold distilled water and centrifuged at 30,000 Xg for 20 min. The top lipid layer was removed and the supernatant decanted. The supernatant was then centrifuged at 100,000 Xg for one hour and the resulting supernate (S_{100}) saved. Twenty mCi [6-^{3}H]N-acetylmannosamine was dried under a stream of N_2 in a 50 ml Erlenmyer flask and dissolved in 5.9 ml of distilled water. Also added to this flask was (a) 3.6 ml of a solution containing 1.6 ml of 1 M Tris-acetate pH 7.6, 60 mg phosphoenolpyruvate, 18 mg ATP, 1 mg NAD^+, and 1.5 mg $NADP^+$, (b) 6.4 ml of liver S_{100} supernate and (c) 100 µl of 1 M magnesium acetate. The total 16 ml of solution was incubated at 37°C for three hours, and then 8 ml of cold (about 5°C) distilled water was added. The resulting suspension was mixed with use of a Vortex and then centrifuged at 400 Xg. The supernate was decanted and diluted to 150 ml with distilled water, yielding a crude solution containing labelled sialic acid.

Purification of [9-^{3}H]sialic acid [149]. Radiolabelled sialic acid was purified by applying the crude solution to Dowex-1 acetate (100-200 mesh, 1.8 x 3.6 cm) column. The column was washed with 800 ml of distilled water (the final column effluent was low in radioactivity), then eluted with a linear gradient of 500 ml 0.1 M to 500 ml 3.0

M acetic acid. Ten ml fractions were collected and 10 µl of each were analyzed for radioactivity. Fractions containing the radioactive peak were pooled, the mixture was lyophilized and 20 ml ethanol:water (7:3, v/v) was added. Purity was determined by silica gel thin-layer chromatography using a solvent system of n-butanol:pyridine:water (6:4:3, v/v). Only one radioactive product was detected and this product moved with the same mobility as authentic sialic acid.

II. Preparation of Lactosylceramide Standard.

Isolation of lactosylceramide [150]. Total lipids were extracted from 1 kg bovine liver using chloroform: methanol (2:1, v/v), the solvent was removed *in vacuo* at 40°C and the lipids were resuspended in chloroform:methanol:water (30:60:8, v/v). This lipid extract was applied to a Sephadex A-25 DEAE column. To elute neutral lipids the column was washed with chloroform:methanol:water (30:60:8, v/v) then methanol. The neutral lipid fraction was dried *in vacuo* at 40°C and mildly saponified [151]. Neutral glycosphingolipids were isolated by Folch partition [152] and applied to an Iatrobeads column [153] (2 x 28 cm). The column was eluted with chloroform:methanol:water (65:25:4, v/v) to separate individual neutral glycosphingolipids. Column fractions were analyzed by thin-layer chromatography, and fractions found to contain lactosylceramide were pooled.

Quantitation of lactosylceramide. Lactosylceramide was quantitated by the phenol-sulfuric acid colorimetric method [154]. The purpose of this was to prepare a working solution of lactosylceramide for enzyme assays. Lactosylceramide (50 µl of the pooled-fractions) was dried under a stream of N_2 and one ml water was added. One ml of

five percent phenol was added to the tube containing lactosylceramide and to tubes containing one ml of standard lactose solutions. After the tube contents were mixed, 5 ml concentrated H_2SO_4 (96 percent) was added. The solutions were vortexed and allowed to cool at room temperature. The absorbances of the solutions were determined at 480 nm and the amount of lactosylceramide determined by comparison with standard lactose solutions. A working solution of lactosylceramide was made to a concentration of 50 nmoles per 50 µl of chloroform:methanol (2:1, v/v).

III. Cell Cultures

Cells were cultured in a humidified 5 percent CO_2-air atmosphere using Corning plastic tissue culture flasks. V79 cells were grown in a modified Eagle's medium (Earle's balanced salt solution with a 50 percent increase of essential amino acids and vitamins, a 100 percent increase of non-essential amino acids and 1 mM sodium pyruvate) [155] supplemented with five percent (v/v) fetal calf serum (FCS). NIL-8 cells were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10 percent (v/v) FCS. Both growth media contained 50 µg/ml gentamycin and 2.5 µg/ml fungizone. Cells were routinely subcultured by washing monolayers with a solution of 0.05 percent trypsin and 0.02 percent EDTA. After removal of the rinse, flasks were placed in an incubator at 37°C for 10 min. Trypsinization was stopped by the addition of medium containing serum, and cells were seeded into new flasks at approximately 2 to 5 x 10^4 cells/cm^2.

IV. Determination of Cell Growth

Two methods were used to characterize cell growth: determination of protein content by the procedure of Lowry et al. [156] and determination of total cell number with a hemacytometer [157]. In growth experiments involving V79 cells, cells were plated at 10^5 cells/cm^2 in 10 x 35 mm tissue culture dishes with 1.5 ml of medium containing 1.0 µg/ml of various phorbol esters:4α-phorbol-12,13-dideca-noate (4α-PDD), phorbol-12,13-dibutyrate (PDB), or phorbol-12-myri-state-13-acetate (PMA). NIL-8 cells were plated at 0.5 x 10^4 cells/cm^2 in 10 x 35 mm tissue culture dishes with 1.5 ml DME containing 10 percent (v/v) FCS (DME/FCS). In both cases, determinations of cell growth were made in triplicate approximately every six hours.

V. Determination of Cell Cycle.

Synchronization of cells. NIL-8 cells in exponential growth phase were synchronized by a double thymidine block followed by colcemid treatment to collect mitotic cells [158]. Cells were initially treated with DME/FCS containing 2 mM thymidine for 16 hours, then medium without thymidine for 8 hours, followed by medium containing 2 mM thymidine for 16 hours. Next, the cells were grown four hours in DME/FCS followed by DME/FCS containing 0.06 µg colcemid per ml medium. Finally two hours after colcemid treatment, mitotic cells were collected at 30 min intervals for six hours by gently shaking culture flasks to suspend mitotic cells. The culture medium was replaced, and the old medium centrifuged at 400 Xg for 5 min to pellet mitotic cells. Pelleted cells were then pooled and kept at 4°C.

Synchronized NIL-8 cells were plated at 2 x 10^4 cells/cm^2 in 10 x 35 mm tissue culture dishes with 1.5 ml medium. The cell cycle was evaluated every two hours by Giemsa staining of mitotic figures [159], evaluating DNA synthesis [157] and determining total cell number [157].

Giemsa staining of mitotic figures. The Giemsa staining solution was made by adding 0.75 g Giemsa stain and 50 ml glycerol to a 200 ml flask and heating the contents of the flask to 56°C for 90-120 min. After cooling, 50 ml of methanol was added and the contents of the flask mixed. After standing at room temperature for seven days, and each time before use, the stock staining solution was filtered. Working Giemsa stain was made by diluting the stock stain ten-fold with hot tap water.

Before application of the Giemsa stain, the monolayer cell cultures were rinsed with cold phosphate buffered saline (PBS), fixed with ethanol for 15 to 30 seconds and air dried. Cells were stained with the working Giemsa solution for 30 min and then rinsed with tap water followed by distilled water. Stained cells were microscopically observed and the number of mitotic cells determined.

Determination of DNA synthesis. To evaluate DNA synthesis, old medium was removed from synchronized NIL-8 cell monolayer cultures and 1 ml of DME/FCS containing 1 µCi [methyl-^{3}H]thymidine added for 30 min. Labelled cells were then washed three times with PBS, drained well and stored at -20°C until the entire set of time points was collected. Monolayers were incubated at 37°C for 10 min with one ml of a solution containing 0.05 percent trypsin and 0.02 percent EDTA. Cells were dislodged from culture dishes, and the entire trypsin solution

with cells was added to prewet 2.4 cm GF/C filters on a multisample vacuum filtration manifold. Culture dishes were washed with one ml PBS and the washings added to the filters. The filters were washed twice with 10 ml of PBS, once with 10 ml of 5 percent TCA and once with 10 ml of methanol. Filters were then transferred to liquid-scintillation vials, allowed to air-dry over night and radioactivity representing DNA synthesis was determined by liquid-scintillation counting.

VI. Labelling of V79 Cell Glycosphingolipids.

Cells were plated in 20 x 100 mm tissue culture dishes at a density of 2 x 10^5 cells/cm^2 in 10 ml of medium containing either 2.0 µCi of [1-^{14}C]palmitate or 0.25 µCi of [9-^{3}H]sialic acid per ml of medium. Labelled compound incorporation, particularly sialic acid, into growth-inhibited V79 cells was extremely low. The above plating density allowed for maximal cell attachment and a six hour active uptake before density-dependent inhibition of cell growth occurred (approximately 2.6 x 10^5 cells/cm^2). At this stage (when density-dependent inhibition of cell growth occurred), the various phorbol esters were added individually to the culture medium to give a 1.6 µM concentration. After 18 hours exposure to the phorbol esters, cells were harvested by scraping with a rubber policeman, washed with PBS and pelleted. Cell labelling was performed with ten plates per treatment (control, 4αPDD, PDB and PMA). The experiment was performed three times to determine mean values for the data.

VII. Glycosphingolipid Determination.

Isolation of glycosphingolipids (GSL) [150]. Initially, total lipids were isolated from cell pellets by extraction with chloroform: methanol (2:1, v/v), dried under a stream of N_2 and resuspended in

chloroform:methanol:water (30:60:8, v/v). The lipid extract was applied to a Sephadex A-25 DEAE column [150,153]. Neutral lipids were eluted with chloroform:methanol:water (30:60:8, v/v) and polar lipids with methanol. The column was then treated with 0.2 M sodium acetate in methanol to elute acidic lipids. Both neutral and acidic lipids were mildly saponified [151] to degrade alkaline-labile phospholipids and neutral glycerides.

Neutral GSL were isolated from the saponified neutral lipid fraction by Folch partition [152]. [1-^{14}C]Palmitate incorporation was determined by liquid-scintillation counting. Total neutral GSL sphingosine levels were determined by the method of Lauter and Trams [160]. Individual neutral GSL were separated by silica gel thin-layer chromatography using a chloroform:methanol:water (60:35:8, v/v) solvent system, eluted from the silica gel with chloroform:methanol (2:1, v/v) and incorporation of radiolabel was determined by liquid-scintillation counting.

The mildly saponified acidic lipid solution was neutralized by the addition of freshly prepared 1 N acetic acid in methanol and extracted three times with hexane to remove protonated fatty acids. The acidic lipid fraction was then dried under a stream of N_2, resuspended in distilled water and dialyzed for 38 hours at 4°C against several changes of water. This ganglioside fraction was analyzed for [1-^{14}C]palmitate and [9-^{3}H]sialic acid incorporation by liquid-scintillation counting. Sphingosine [160] and lipid-bound sialic acid [161] levels of gangliosides were determined by standard methods. Individual gangliosides were separated by silica gel thin-layer chromatography using a chloroform:methanol:aqueous 0.02 percent $CaCl_2$

(50:40:9, v/v) solvent system. They were eluted from the silica gel with chloroform: methanol (2:1, v/v) and the incorporation of radiolabel was determined by liquid-scintillation counting.

Quantitation of glycosphingolipids. Sphingosine levels of neutral GSL and gangliosides were determined by the spectrophotometric method of Lauter and Trams [160]. Samples to be analyzed were dried under a stream of N_2, redissolved in 0.6 ml of 0.01 N HCl, then made alkaline by adding 1.4 ml of 0.01 N NaOH. Next, 5 ml of ethyl acetate was added, the solution was vortexed and then was centrifuged at 400 Xg for 10 min. The ethyl acetate phase was removed and washed twice with two ml of water by vortexing and centrifuging. Two ml of 0.01 M acetate buffer pH 3.65 (previously washed with ethyl acetate) was added to the washed ethyl acetate phase along with 0.1 ml of methyl orange reagent. The resulting solution was vortexed for one min and then was centrifuged at 400 Xg for 10 min. Methyl orange reagent was made by dissolving 500 mg of methyl orange in 100 ml of warm distilled water and washing three times with 50 ml of chloroform. The absorbance of the sphingosine-methyl orange complex in the ethyl acetate supernatant phase was determined at 415 nm and compared to the absorbance of identically treated standards containing 0.01 to 0.1 μmoles of sphingosine. All absorbances were determined against blanks carried through the same procedure.

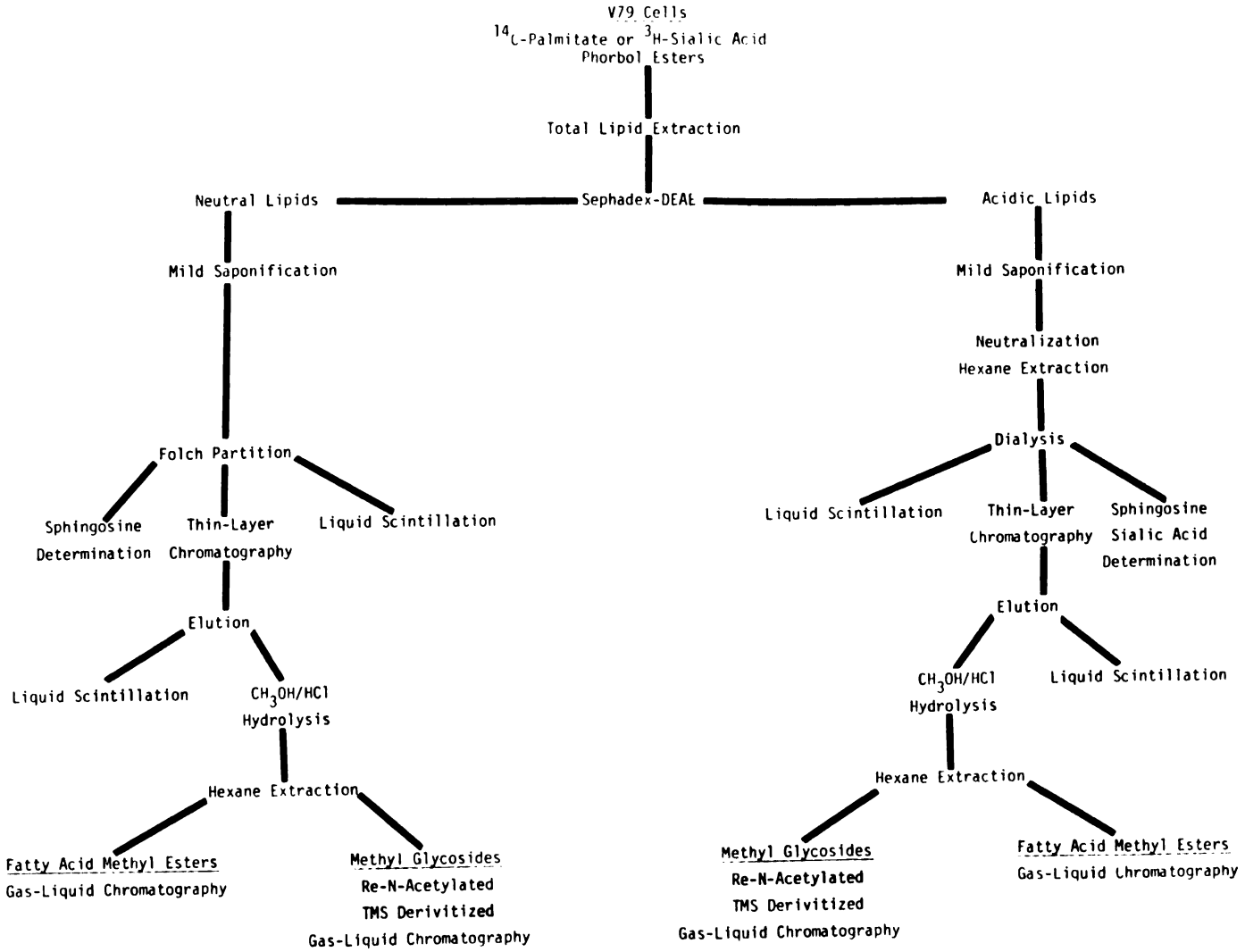
Lipid-bound sialic acid determination. Lipid-bound sialic acid levels of gangliosides were determined by the resorcinol colorimetric method [161]. Samples to be analyzed were dried under a stream of N_2 in 13 x 100 mm screw cap test tubes, 250 μl of distilled water and 250 μl of resorcinol reagent were added and the mixture was vortexed.

Resorcinol stock solution was made by dissolving 2.0 g resorcinol in 100 ml of water. Resorcinol reagent was made prior to use by mixing 10 ml of resorcinol stock solution with 80 ml of concentrated HCl, 250 µl of 0.1 M $CuSO_4$ and 10 ml of water. Samples treated with resorcinol reagent were incubated at 100°C for 20 min in the capped tubes and then placed in an ice bath. After the samples were cold, 0.5 ml of n-butyl acetate:n-butanol (85:15) was added to each, the solutions were vortexed and then centrifuged at 800 Xg for one min. The absorbance of the upper layer of the resulting partition was determined at 580 and 450 nm, and compared to the absorbance of identically treated sialic acid and galactose standards. Hexose absorbance was corrected for as described [162]. Sample blanks were carried through the same procedure, but contained no resorcinol. This was done to eliminate absorbance not due to sialic acid complexing with resorcinol.

Determination of the fatty acid composition of glycosphingolipid. Individual neutral GSL and gangliosides eluted from silica gel with chloroform:methanol (2:1, v/v) were dried under a stream of N_2 in a screw cap test tube with teflon-lined cap, three ml of methanol:acetyl chloride (20:1, v/v) was added and the solutions were allowed to stand tightly capped at 80°C for 15 hours (overnight). The samples were cooled to room temperature and two ml of hexane were added. After vortexing and centrifuging at 400 Xg for 10 min, the upper hexane phase containing fatty acid methyl esters was removed. The lower methanol phase was washed twice with two ml hexane, and the washings pooled with the upper hexane phase. The pooled solution was dried under a stream of N_2 and the fatty acid methyl esters were taken up in 0.5 ml hexane then analyzed by gas-liquid chromatography. The fatty acid methyl

Figure 4. Determination of glycosphingolipid carbohydrate and fatty acid composition.

DETERMINATION OF GLYCOSPHINGOLIPID COMPOSITION



esters were separated using a column containing fifteen percent DEGS (diethylene glycol succinate) on 100-200 mesh Gas-Chrom Q at 170°C.

Determination of the carbohydrate composition of glycosphingolipid. The methanol layer after hexane extraction was neutralized by gradually adding ten to fifty mg of Ag_2CO_3 powder with frequent vortexing. When the methanol solution containing methyl glycosides was at pH 6 by litmus paper test, 300 µl of acetic anhydride was added and the mixture allowed to stand at room temperature for eight to twelve hours to re-N-acetylate amino sugars. The mixture was centrifuged for five min at 400 Xg and the supernate transferred into a one dram vial, and the solvent subsequently evaporated under a stream of N_2. The trimethylsilyl ether (TMS) derivatives of the methyl glycosides were prepared by the addition of 0.5 ml of TMS derivitizing solution. The TMS derivitizing solution was made by mixing 18 ml of freshly distilled dry pyridine (bp 115°C), six ml of hexamethyldisilazane and two ml of trimethylchlorosilane. The samples were allowed to stand at room temperature for 30 min to form the derivatives, and analyzed by gas-liquid chromatography. The TMS derivatives of the methyl glycosides were separated using a column containing three percent SE-30 (methylsilicone) with a temperature gradient of 140-240°C at 3°C per min [163].

VIII. CMP-Sialic Acid:Lactosylceramide Sialyltransferase Assay.

Preparation of cell homogenates. Cultured cells were washed twice with PBS followed by incubation at 37°C with 2 mM EDTA in PBS. Cells were removed from the culture dish surfaces by vigorous shaking and collected by centrifugation. The pelleted whole cells were stored

frozen with one volume of 0.32 M sucrose at -20°C. Prior to assays, cells were thawed and sonicated for 10 sec. The homogenates were used directly for transferase assays. The protein concentration of homogenates was determined by the method of Lowry et al. [156] using crystalline bovine serum albumin as a standard.

Determination of enzyme activity. The assay mixtures contained the following components in 50 or 100 µl: 0.5 mM lactosylceramide, 0.4 mM CMP-[4,5,6,7,8,9-^{14}C]sialic acid (15 x 10^6 dpm/µmole), 50 mM cacodylate-HCl buffer pH 6.5, 15 mM $MnCl_2$, 0.3 percent (w/v) Triton CF-54, and 0.2 to 0.6 mg protein from crude cellular homogenates. Assays without added lactosylceramide were used as blanks and the resulting values were substracted from all data points. Studies of inhibition of CMP-sialic acid:lactosylceramide sialyltransferase activity by potential glycoconjugate inhibitors were performed by including various amounts of the glycoconjugates in assay mixtures. Assays were run for sixty min. at 37°C and terminated by the addition of 25 µl of ethanol.

Assay tubes were prepared by pipetting both lactosylceramide in chloroform:methanol (2:1, v/v) and radiolabelled CMP-sialic acid in ethanol: water (7:3, v/v) into 10 x 75 mm test tubes and drying under a stream of N_2. Next 25 or 50 µl of assay buffer (100 mM cacodylate-HCl pH 6.5, 0.6% Triton CF-54, and 30 mM $MnCl_2$) and 25 or 50 µl of crude cellular homogenates were added.

The reaction products were analyzed by a double paper chromatographic method [164]. Samples were applied in two cm lanes 26 cm from the top of a 20 x 55 cm sheet of Whatman No. 4 chromatography paper. After the aqueous samples were applied, assay tubes were rinsed twice with 50

µl of chloroform:methanol (2:1, v/v) and these rinses applied to the same spots. Papers were developed (descending) with one percent NaB_4O_7 overnight. After drying, the chromatograms were cut off three cm below the origin and the origin-containing sections were developed (ascending) with n-propanol:water (7:3, v/v). Each lane of these chromatograms was cut into two cm sections and the radioactivity of each section determined by liquid-scintillation counting. Counts of all sections (excluding the origin) for each lane were totaled. The counts for an endogenous acceptor control (no lipid acceptor added) were substracted to give net radioactivity. These counts were converted to pmoles from the known specific activity of radiolabelled sialic acid. In experiments where many samples were analyzed, only descending chromatographic development with one percent NaB_4O_7 was done and the origin counted [35]. No difference in net radiolabelled sialic acid incorporation was seen when mono-directional and bi-directional chromatographic development was performed on the same samples.

Various buffers and detergents were used in the sialyltransferase assays to determine the optimum conditions for transferase activity. In addition, phospholipids of different structures were used in assays of the solubilized enzyme to determine their effect on activity.

IX. α-Galactosidase A Activity.

The assay was modified from the method of Dean and Sweeley [26]. Assay mixtures of 200 µl total volume contained: 4.3 mM 4-methylumbelliferylα-D-galactoside, 50 mM N-acetylgalactosamine, Gomori citrate-phosphate buffer pH 4.6 [165], and 60 to 120 µg crude cellular homogenates. Assays were run for five and ten min at 37°C, and the

reactions terminated by the addition of 2.35 ml of 0.1 M ethylenediamine, pH 11. Fluorescence was determined and compared to standard 4-methylumbelliferone in 0.1 M ethylenediamine.

X. Isolation of Golgi membranes.

The standard fractionation procedure used was that of Morre et al. [166,167] and Schachter et al. [168]. All steps were carried out at 5°C. Fifteen grams of chicken liver were minced thoroughly with a razor blade and placed directly into a 50 ml centrifuge tube containing 15 ml of 0.5 M sucrose in Golgi grind buffer (100 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$ and 1% dextran). The tissue was homogenized for 40 sec on a Polytron operating at the lowest speed, while moving the tube up and down to guide pieces of tissue into the homogenizing probe. The homogenate was mixed with 15 ml of 0.5 M sucrose in Golgi grind buffer and centrifuged at 3020 Xg (5,000 rpm) for 15 min in a Sorval SS-34 rotor to remove large bits of tissue. The resulting supernatant fraction was mixed with 30 ml of 0.5 M sucrose in Golgi grind buffer.

Ten ml of homogenate (approximately two grams of original tissue at one gram of tissue per four ml of homogenate) was applied to the tops of discontinuous sucrose density gradients in 25 ml tubes (10 ml of 1.2 M sucrose and 5 ml of 0.7 M sucrose, both in Golgi grind buffer) and centrifuged at 105,000 Xg (37,000 rpm) in a Sorval Ti70 rotor for 90 min. After centrifugation, the fluffy membranous material at the interface between the 0.7 M and 1.2 M sucrose layers was isolated, diluted four-fold with cold distilled water and pelleted at 50,000 Xg (26,000 rpm) in a Sorval Ti70 rotor for 15 min. The supernatant

fraction was decanted and the pelleted Golgi membranes removed with a spatula.

XI. Detergent Extraction of Golgi Membranes.

All steps in the solubilization of CMP-sialic acid:lactosylceramide sialyltransferase from Golgi membranes were done at 5°C. Pelleted Golgi membranes were deposited into 20 ml of Golgi membrane extraction buffer (25 mM Tris-HCl buffer pH 6.5 containing 20 mM $MnCl_2$, 0.6% Triton X-100 and 40% glycerol), and then sonicated four times for 10 sec with 30 sec intervals. The suspension containing disrupted Golgi membranes was magnetically stirred for one hour, then pelleted at 150,000 Xg (45,000 rpm) in a Sorval Ti70 rotor for 90 min. The supernate containing sialyltransferase activity was decanted and stored at -20°C. This preparation was termed solubilized sialyltransferase.

XII. Partial Purification of CMP-Sialic Acid:Lactosylceramide Sialyltransferase.

Solubilized sialyltransferase was applied to a 1.5 x 16 cm cellulose-phosphate column previously equilibrated with the Golgi membrane extraction buffer and eluted with the same buffer. One ml fractions were collected and analyzed for sialyltransferase activity. Fractions with activity were pooled.

Pooled fractions were then applied to a Sephadex G-150 column previously equilibrated with the Golgi membrane extraction buffer and eluted with the same buffer. One ml fractions were collected and analyzed for sialyltransferase activity. Fractions with transferase activity were pooled and stored at -20°C. This preparation was termed the partially purified sialyltransferase.

XIII. Treatment of Solubilized CMP-Sialic Acid:Lactosylceramide Sialyltransferase with Phosphatase.

Two ml of solubilized sialyltransferase was incubated at 37°C with 100 µl of phosphatase (1 mg/ml) made up in the Golgi membrane extraction buffer. Incubations were done at pH 6.5 in the extraction buffer. Attempts to change the pH to 8 resulted in the sialyltransferase preparation turning brown and cloudy, and almost total loss of activity upon return to pH 6.5. The type and amount of phosphatase was varied, as was the time of incubation. In addition, the potential phosphatase inhibitors, sodium fluoride and sodium phosphate (pH 6.5) were added to some phosphatase incubations at a 50 mM concentration.

Phosphatase assay. Phosphatase assay mixtures of two ml contained 10 mM p-nitrophenyl phosphate and various concentrations of phosphatase inhibitors (sodium fluoride and sodium phosphate) in extraction buffer. Assays were initiated by the addition of phosphatase (100 µl at 4 mg/ml of extraction buffer) to assay mixtures and terminated by the addition of 100 µl of 100 percent TCA. The solution was made basic by the addition of 200 mM sodium borate (approx. pH 9.5) and the solution absorbance determined at 410 nm. The solution absorbance was compared to that of a standard (1 mM p-nitrophenol in extraction buffer).

XIV. Treatment of Partially Purified CMP-Sialic Acid:Lactosylceramide Sialyltransferase with Protein Kinase.

Partially purified sialyltransferase (1.3 ml) was mixed with 100 µl of several factors and the extraction buffer to give a final volume of 2.0 ml. This mixture was incubated for one hour at 37°C and then used in standard sialyltransferase assays. The final concentration of

added components was 5 mM $MgCl_2$, 1.5 mM ATP, 10 mM phosphocreatine, 40 U/ml creatine phosphokinase, 10 μM cAMP, and 3 U/ml cAMP dependent protein kinase. Individual and combinations of components were deleted from several incubations. Protein kinase inhibitor was used in several assays, also at a concentration of 8.7 U/ml.

RESULTS

I. The Effect of Phorbol Esters on V79 Cell Growth.

The three phorbol esters used in experiments with V79 cells included a powerful tumor-promoter, phorbol-12-myristate-13-acetate (PMA), a moderately active tumor-promoter phorbol-12,13-dibutyrate (PDB), and an inactive compound 4α-phorbol-12,13-didecanoate (4α-PDD). The results presented in Figure 5 indicate that V79 cell growth in the usual exponential phase was not affected by the presence of phorbol esters. Density-dependent inhibition of cell growth occurred at the same density for controls and 4α-PDD treated cells. However, growth inhibition of cells treated with tumor-promoting phorbol esters required appreciably higher densities than for controls. Furthermore, differences in the final saturation density of the cells reflected the *in vivo* phorbol ester tumor-promoting capacity.

II. Effect of Phorbol Esters on V79 Cell Glycosphingolipids

The data in Tables 3 and 4 demonstrate that the presence of tumor-promoting phorbol esters in the cell culture medium of V79 cells resulted in an increase in both [1-^{14}C]palmitate and [9-^{3}H]sialic acid incorporation into gangliosides, plus increased ganglioside sphingosine and sialic acid levels. As with cell saturation density, the increased incorporation of palmitate and sialic acid above that induced by 4α-PDD reflected an increasing progression in phorbol ester *in vivo* tumor-promoting activity. The results also indicate that

Figure 5. The effect of phorbol esters on V79 cell growth as determined two ways: (a) Total cell number; (b) protein assay. Estimations of cell growth were made in triplicate. 4α-PDD, 4α-phorbol-12,13-didecanoate; PDB, phorbol-12,13-dibutyrate; PMA, phorbol-12-myristate- 13-acetate.

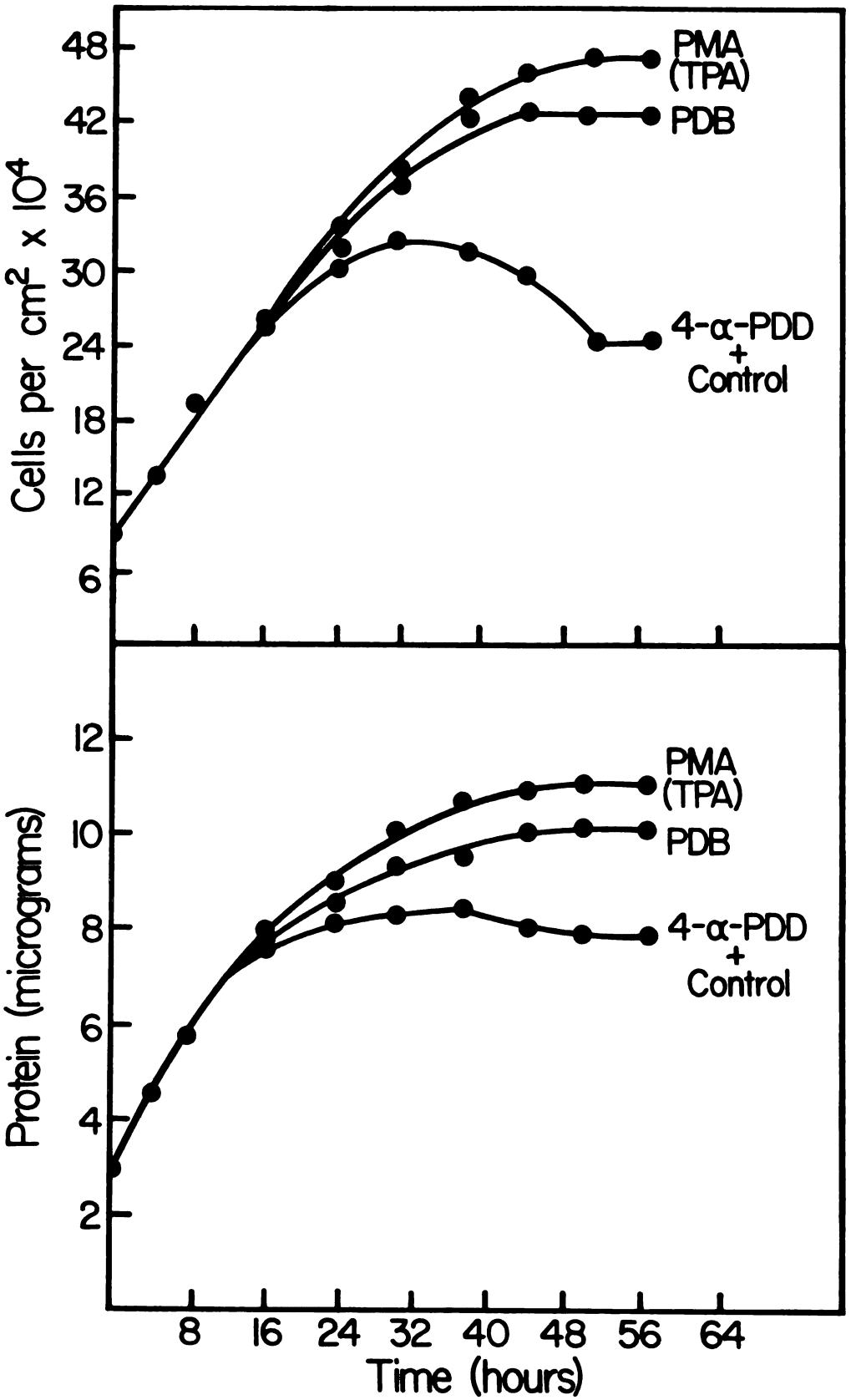

Cells per cm² x 10⁴
48
42
36
30
24
18
12
6
PMA (TPA)
PDB
4-α-PDD + Control
Protein (micrograms)
12
10
8
6
4
2
PMA (TPA)
PDB
4-α-PDD + Control
8
16
24
32
40
48
56
64
Time (hours)

Table 3. The effect of phorbol esters on [1-^{14}C]palmitate and [9-^{3}H]sialic acid incorporation into V79 cell glycosphingolipids

Phorbol esters		Gangliosides		Neutral glycosphingolipid
Compound	Tumor-promoting activity[a]	[1-^{14}C]Palmitate incorporation[b] (cpm/μg protein)	[9-^{3}H]Sialic acid incorporation[b] (cpm/μg protein)	[1-^{14}C]Palmitate incorporation[b] (cpm/μg protein x 10^{-3})
None (Control)	0	48 ± 3.1	7.3 ± 0.6	1.3 ± 0.1
4α-PDD	0	84 ± 5.6	8.5 ± 0.6	1.5 ± 0.1
PDB	2	110 ± 7.6	12.0 ± 1.1	1.3 ± 0.2
PMA	4	160 ± 8.5	21.0 ± 1.5	1.4 ± 0.2

[a] Tumor-promoting potential was rated on a scale of 0-4, with 0 representing no significant activity and 4 the greatest activity [169].
[b] Values represent the mean of three determinations.

Table 4. The effect of phorbol esters on V79 cell glycosphingolipid levels

	Gangliosides		Neutral glycosphingolipid
Phorbol esters	Lipid bound sialic acid[a] (nmoles/mg protein)	Sphingosine[a] (nmoles/mg protein)	Sphingosine[a] (nmoles/mg protein)
None (Control)	1.5 ± 0.1	1.7 ± 0.2	3.7 ± 0.3
4a-PDD	2.1 ± 0.2	2.4 ± 0.2	3.5 ± 0.3
PDB	2.8 ± 0.3	3.0 ± 0.2	3.9 ± 0.3
PMA (TPA)	3.4 ± 0.3	3.5 ± 0.3	3.7 ± 0.3

[a] Values represent the mean of three determinations.

phorbol esters did not induce changes in the sphingosine levels of neutral GSL or in [1-^{14}C]palmitate incorporation into neutral GSL. Table 5 contains data indicating that the V79 cells contain a simple ganglioside composition, the majority of which is G_{M3}. The presence of phorbol esters in the culture medium did not alter the distribution of [1-^{14}C]palmitate or [9-^{3}H]sialic acid label into the individual gangliosides or neutral GSL. Analyses of fatty acids and carbohydrates in gangliosides and neutral GSL, Tables 5 to 17, indicate that V79 cells contain four major neutral GSL, monohexosylceramide (probably GLcCer), dihexosylceramide (probably LacCer), trihexosylceramide (probably Gb3) and tetrahexosylceramide (probably Gb4), in addition to two gangliosides G_{M3} and G_{M2}. The data also indicate that there was no change due to the phorbol ester treatment in the proportion of GSL-bound fatty acids and carbohydrates. Taken together these data suggest that the phorbol esters in the culture medium led to an enhanced level of gangliosides (mostly G_{M3}), due to increased ganglioside biosynthesis, decreased degradation or both. In addition phorbol, ester treatment did not result in any structural changes of GSL.

III. Characterization of an Assay for CMP-Sialic Acid:Lactosylceramide Sialyltransferase.

The effect of the protein concentration of the homogenate on sialyltransferase activity in NIL-8 cell homogenates is shown in Figure 6. Transferase activity increased linearly with homogenate protein concentration from 50 to 700 µg per 50 µl of assay mixture, and decreased at protein concentrations greater than 1100 µg protein per 50

Table 5. The effect of phorbol esters on the percent distribution of [1-^{14}C]palmitate and [9-^{3}H]sialic acid into glycosphingolipids.

Glycosphingolipid	[1-^{14}C]Palmitate (% distribution)[a]				[9-^{3}H]Sialic acid (% distribution)[a]			
	Control	4α-PDD	PDB	PMA	Control	4α-PDD	PDB	PMA
Total ganglioside	100	100	100	100	100	100	100	100
G_{M3}	95 ± 4	96 ± 5	96 ± 5	96 ± 5	93 ± 6	94 ± 5	94 ± 6	93 ± 6
G_{M2}	5 ± 0.1	4 ± 0.1	4 ± 0.2	4 ± 0.2	7 ± 6	6 ± 5	6 ± 6	7 ± 6
Total neutral glycosphingolipid	100	100	100	100				
CMH	51 ± 5	49 ± 4	43 ± 3	42 ± 3				
CDH	13 ± 2	12 ± 3	10 ± 1	17 ± 2				
CTH	2 ± 0.1	4 ± 0.1	3 ± 0.1	2 ± 0.1				
Glob	34 ± 3	35 ± 4	44 ± 4	39 ± 4				

a Values represent the mean of three determinations. G_{M3}, II^{3}-a-N-acetylneuraminyl-lactosylceramide; G_{M2}, II^{3}-a-N-acetylneuraminylgangliotriasylceramide; CMH, monohexosylceramide; CDH, dihexo-sylceramide; CTH, trihexosylceramide; Glob, tetrahexosylceramide.

Table 6. Molar carbohydrate ratios of the acidic glycolipid designated as ganglioside, G_{M3}, based on glucose = 1.00.[a]

Sugar	Control	4αPDD Treated	PDB Treated	PMA Treated
GLC	1.0	1.0	1.0	1.0
Gal	0.9	1.0	0.9	1.1
GlcNAc	nd[b]	nd	nd	nd
GalNAc	nd	nd	nd	nd
NeuAc	0.9	0.9	0.9	0.8

[a] Values represent the mean of three determinations.

[b] Carbohydrate not detected.

Table 7. Fatty acid percent composition of the acidic glycolipid designated as ganglioside G_{M3}.[a]

Fatty acid	Control Percent	4αPDD Percent	PDB Percent	PMA Percent
16:0	18	18	14	17
16:1	1.2	1	2.7	3.3
18:0	42	45	42	41
18:1	33	36	34	33
18:2	0.9	0.8	0.9	1.0
18:3	0.8	0.6	1.4	1.3
20:0	0.9	0.3	1.7	1.1
20:1	0.7	0.7	0.8	0.6

[a] Values represent the mean of three determinations.

Table 8. Molar carbohydrate ratios of the acidic glycolipid designated as ganglioside G_{M2}, based on glucose = 1.00.[a]

Sugar	Control	4αPDD Treated	PDB Treated	PMA Treated
GLC	1.0	1.0	1.0	1.0
Gal	1	0.9	0.9	1.0
GlcNAc	nd[b]	nd	nd	nd
GalNAc	0.9	1.0	1.1	0.9
NeuAc	1.2	1.2	0.9	1.0

[a] Values represent the mean of three determinations.

[b] Carbohydrate not detected.

Table 9. Fatty acid percent composition of the acidic glycolipid designated as ganglioside G_{M2}.[a]

Fatty acid	Control Percent	4αPDD Percent	PDB Percent	PMA Percent
16:0	16	18	20	17
16:1	1.2	1.1	1.8	1.6
18:0	45	44	40	42
18:1	32	32	33	34
18:2	0.9	0.9	0.8	0.9
18:3	0.8	0.7	0.8	0.8
20:0	0.9	0.6	0.7	0.9
20:1	0.6	0.7	0.8	0.7

[a] Values represent the mean of three determinations.

Table 10. Molar carbohydrate ratios of the neutral glycolipid designated as monohexosylceramide, based on glucose = 1.00.[a]

Sugar	Control	4αPDD Treated	PDB Treated	PMA Treated
GLC	1.0	1.0	1.0	1.0
Gal	0.2	0.1	0.1	0.1
GlcNAc	nd[b]	nd	nd	nd
GalNAc	nd	nd	nd	nd
NeuAc	nd	nd	nd	nd

[a] Values represent the mean of three determinations.

[b] Carbohydrate not detected.

Table 11. Fatty acid percent composition of the neutral glycolipid designated as monohexosylceramide.[a]

Fatty acid	Control Percent	4αPDD Percent	PDB Percent	PMA Percent
16:0	16	19	19	18
16:1	1.1	1.1	1.7	1.1
18:0	44	43	42	44
18:1	33	32	32	32
18:2	1.0	0.9	1.0	0.9
18:3	0.8	0.9	1.0	0.9
20:0	0.9	0.7	0.8	0.6
20:1	0.6	0.6	0.7	0.7

[a] Values represent the mean of three determinations.

Table 12. Molar carbohydrate ratios of the neutral glycolipid designated as dihexosylceramide, based on glucose = 1.00.[a]

Sugar	Control	4αPDD Treated	PDB Treated	PMA Treated
GLC	1.0	1.0	1.0	1.0
Gal	0.8	0.9	0.8	0.9
GlcNAc	nd[b]	nd	nd	nd
GalNAc	nd	nd	nd	nd
NeuAc	nd	nd	nd	nd

[a] Values represent the mean of three determinations.

[b] Carbohydrate not detected.

Table 13. Fatty acid percent composition of the neutral glycolipid designated as dihexosylceramide.[a]

Fatty acid	Control Percent	4αPDD Percent	PDB Percent	PMA Percent
16:0	71	19	19	18
16:1	1.4	1.1	1.2	1.4
18:0	42	43	41	42
18:1	34	32	33	33
18:2	1.1	1.0	1.3	1.1
18:3	0.8	0.9	0.9	0.8
20:0	0.7	0.7	0.8	0.6
20:1	0.6	0.6	0.6	0.6

[a] Values represent the mean of three determinations.

Table 14. Molar carbohydrate ratios of the neutral glycolipid designated as trihexosylceramide, based on glucose = 1.00.[a]

Sugar	Control	4αPDD Treated	PDB Treated	PMA Treated
GLC	1.0	1.0	1.0	1.0
Gal	1.7	1.7	1.7	1.7
GlcNAc	nd[b]	nd	nd	nd
GalNAc	nd	nd	nd	nd
NeuAc	nd	nd	nd	nd

[a] Values represent the mean of three determinations.

[b] Carbohydrate not detected.

Table 15. Fatty acid percent composition of the neutral glycolipid designated as trihexosylceramide.[a]

Fatty acid	Control Percent	4αPDD Percent	PDB Percent	PMA Percent
16:0	17	16	18	17
16:1	1.0	1.1	1.1	1.5
18:0	43	41	45	42
18:1	34	37	32	34
18:2	0.8	0.9	0.7	0.9
18:3	0.8	0.9	0.7	0.8
20:0	0.8	0.7	0.7	0.7
20:1	0.6	0.7	0.6	0.6

[a] Values represent the mean of three determinations.

Table 16. Molar carbohydrate ratios of the neutral glycolipid designated as globoside, based on glucose = 1.00.[a]

Sugar	Control	4αPDD Treated	PDB Treated	PMA Treated
GLC	1.0	1.0	1.0	1.0
Gal	1.7	1.7	1.7	1.7
GlcNAc	nd[b]	nd	nd	nd
GalNAc	0.8	0.8	0.8	0.8
NeuAc	nd	nd	nd	nd

[a] Values represent the mean of three determinations.

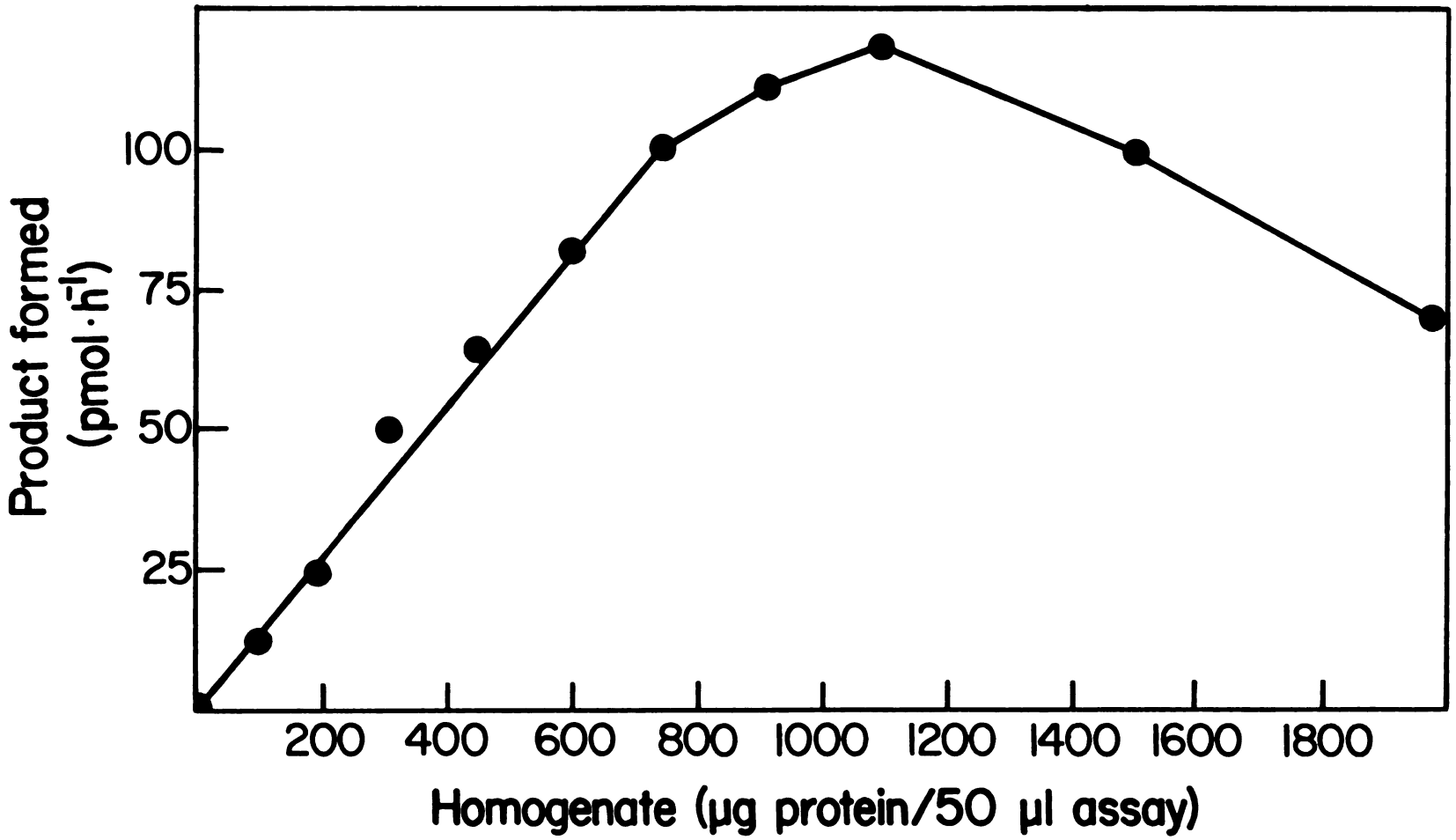
[b] Carbohydrate not detected.

Table 17. Fatty acid percent composition of the neutral glycolipid designated as globoside.[a]

Fatty acid	Control Percent	4αPDD Percent	PDB Percent	PMA Percent
16:0	17	19	17	18
16:1	1.3	1.2	1.8	1.3
18:0	41	44	43	46
18:1	36	31	34	30
18:2	0.9	0.8	0.9	0.9
18:3	0.7	0.7	0.8	0.7
20:0	0.8	0.8	0.7	0.7
20:1	0.6	0.6	0.7	0.6

[a] Values represent the mean of three determinations.

Figure 6. Effect of the protein concentration of the NIL-8 cell homogenate on sialyltransferase activity. Varying amounts of crude cell homogenates were added to the reaction mixture as described in Methods.


Product formed
(pmol·h⁻¹)
100
75
50
25
200
400
600
800
1000
1200
1400
1600
1800
Homogenate (µg protein/50 µl assay)

μl. This decrease in activity may have been due to a decreased detergent-to-protein ratio, since additional detergent resulted in increased enzyme activity in assays with high protein concentration. In other studies, it has been observed that detergent solubilization correlated with the detergent-to-membrane protein ratio, particularly if the concentration of the membrane preparation is relatively high [170].

The effect of incubation time on NIL-8 cell sialyltransferase activity is shown in Figure 7. Sialyltransferase activity was linear with time from 15 min to 4.0 hours; at longer times activity deviated from linearity. One hour was routinely used to measure transfer of labeled NeuAc to exogenous lactosylceramide. This enzyme activity appears to be relatively stable compared to other glycosyltransferase activities in crude cellular homogenates [171].

The substrate-binding constants for CMP-sialic acid and lactosylceramide were determined as shown in Figures 8 and 9, respectively. The data were plotted as reciprocals of velocity and substrate concentration, and $K_{m,app}$ and V_{max} values were calculated by a weighted linear least-squares analysis as suggested by Wilkinson [172]. The $K_{m,app}$ values for both CMP-sialic acid, 0.16 mM, and lactosylceramide, 0.11 mM, are similar to those described for other glycolipid glycosyltransferases [171,173]. The V_{max} of the enzyme was determined to be 112 pmoles/mg protein/hr. This is somewhat lower than for NIL-8 cell glycolipid β-N-acetylgalactosaminyltransferase [171].

Figure 10 shows the effect of Mn^{+2}, Mg^{+2}, and Ca^{+2} on NIL-8 sialyltransferase activity. Without added metal ion the activity

Figure 7. Effect of incubation time on sialyltransferase activity. Crude homogenates were incubated with the reaction mixtures for varying amounts of time as described in Methods.

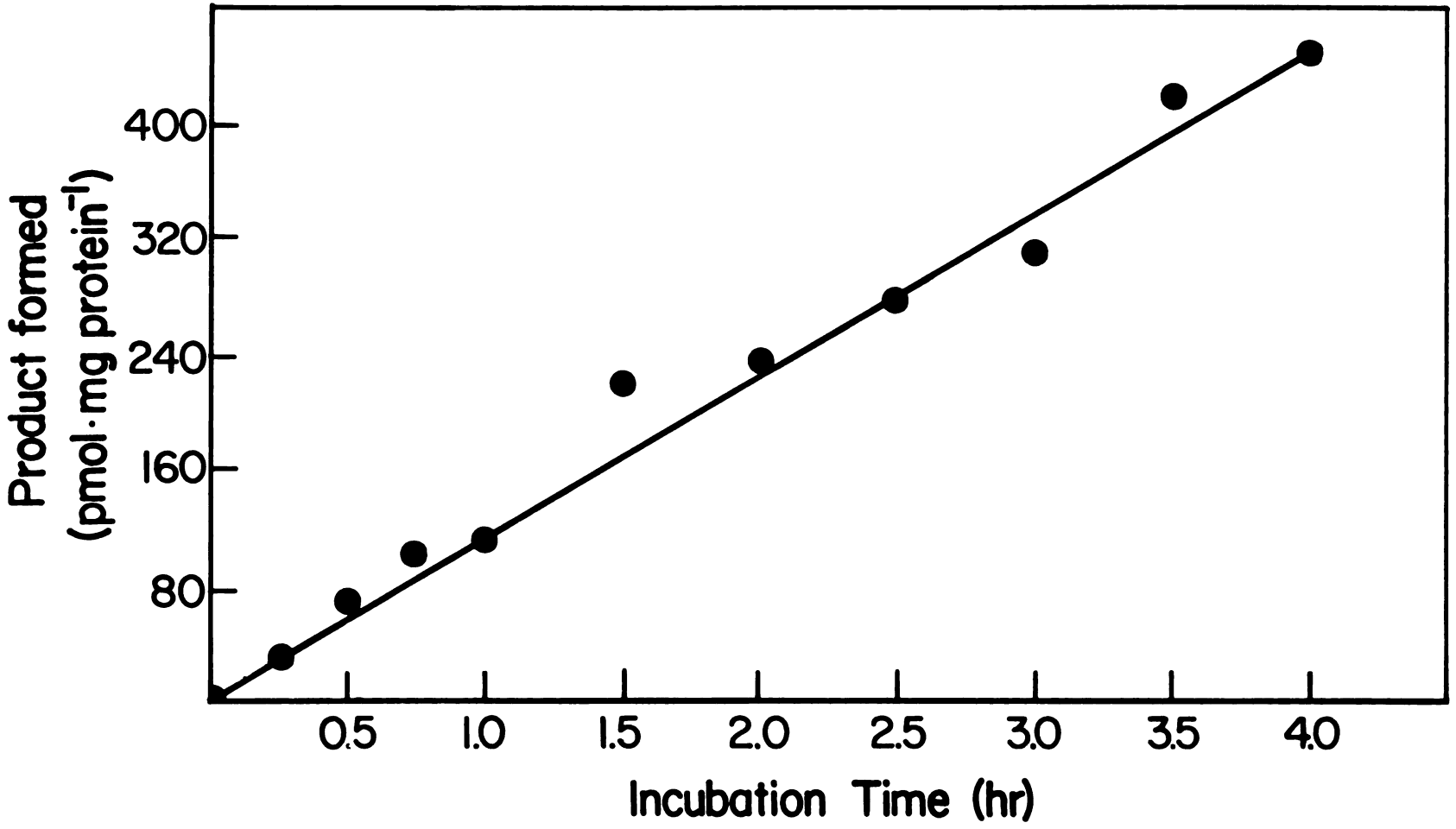

Product formed
(pmol·mg protein⁻¹)
400
320
240
160
80
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
Incubation Time (hr)

Figure 8. Effect of cytidine monophosphate sialic acid (CMP-sialic acid) concentration on sialyltransferase activity in NIL-8 cell homogenates. Various concentrations of CMP-sialic acid (constant specific activity) were added to reaction mixtures. An estimation of the $K_{m,app}$ for CMP-sialic acid was obtained by an analysis of double-reciprocoal V vs. S plots (inset).

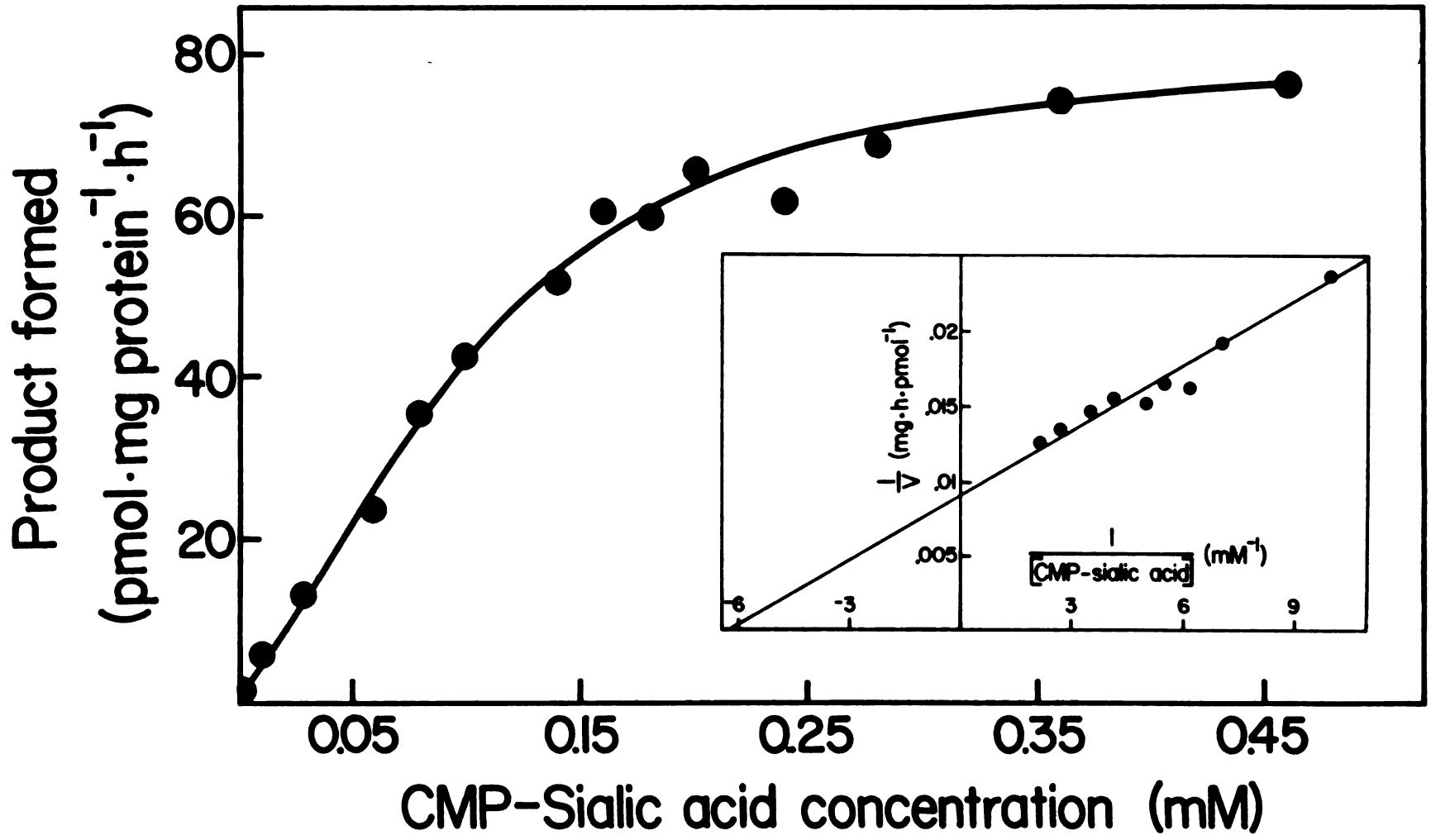

Product formed
(pmol·mg protein⁻¹·h⁻¹)
80
60
40
20
0.05
0.15
0.25
0.35
0.45
CMP-Sialic acid concentration (mM)
1/V (mg·h·pmol⁻¹)
.02
.015
.01
.005
1
[CMP-sialic acid] (mM⁻¹)
-6
-3
3
6
9

Figure 9. Effect of lactosylceramide concentration on sialyltransferase activity in NIL-8 cell homogenate. Various concentrations of exogenous lactosylceramide acceptor were added to reaction mixtures. An estimation of the $K_{m,app}$ for lactosylceramide was obtained by an analysis of double reciprocal V vs. S plots (inset).

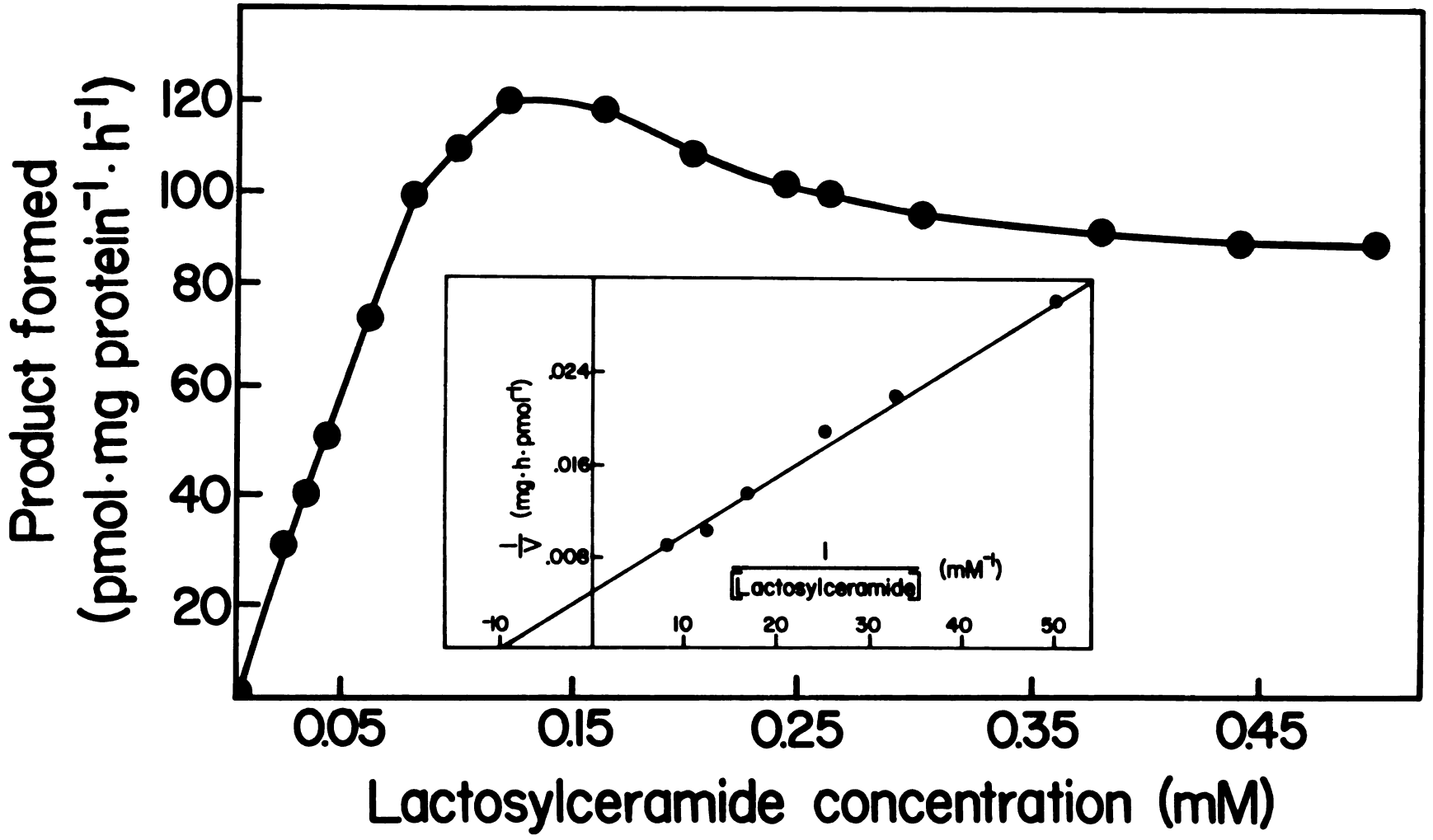
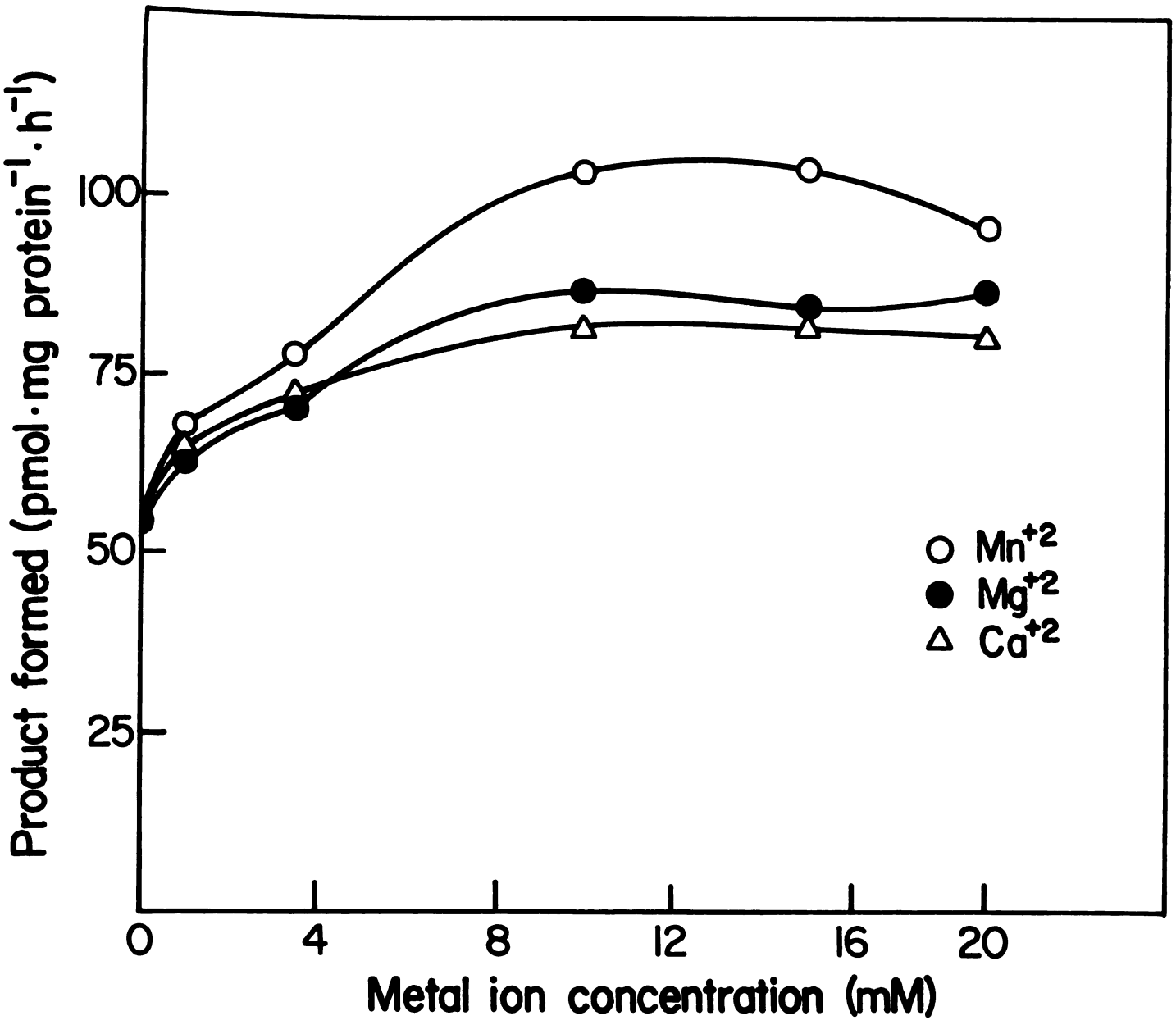

Product formed
(pmol·mg protein⁻¹·h⁻¹)
120
100
80
60
40
20
0.05
0.15
0.25
0.35
0.45
Lactosylceramide concentration (mM)
1/V (mg·h·pmol⁻¹)
.024
.016
.008
-10
10
20
30
40
50
1/[Lactosylceramide] (mM⁻¹)

Figure 10. Effect of divalent metal cation concentrations on sialyltransferase activity in NIL-8 cell homogenates. Varying concentrations of Mn^{2+} (O), Mg^{2+} (●), or Ca^{2+} (Δ) were added to reaction mixtures and the transferase activity determined as described in Methods.


Product formed (pmol·mg protein^{-1}·h^{-1})
100
75
50
25
0
4
8
12
16
20
Metal ion concentration (mM)
Mn^{+2}
Mg^{+2}
Ca^{+2}

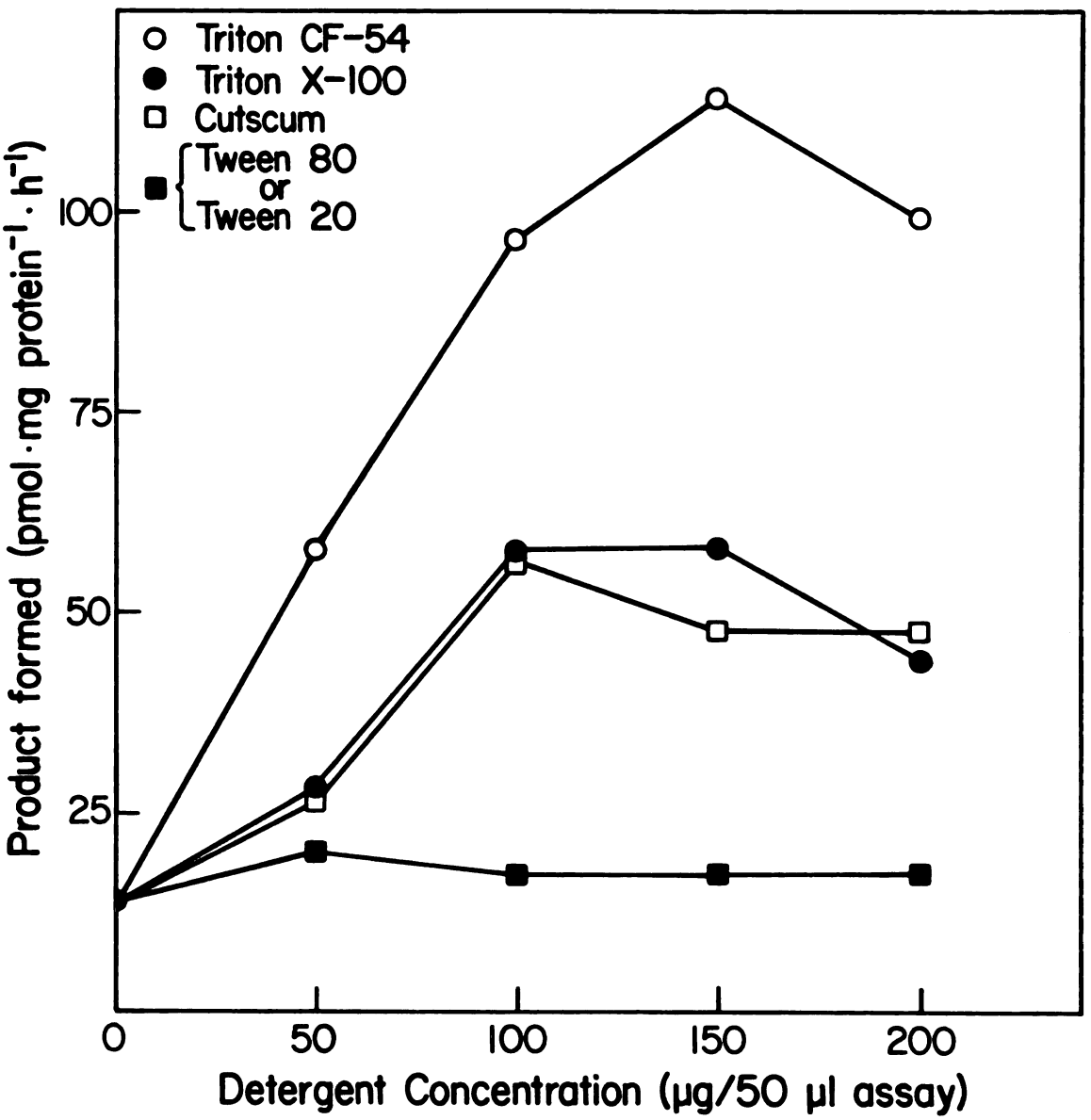
of the enzyme was about fifty percent of the maximum level. Manganese gave a maximum activation of transferase activity at 10 mM. Magnesium and calcium were slightly less effective in activating at 10 mM. Magnesium and calcium were slightly less effective in activating the transferase at all concentrations.

Various types of detergent, anionic (sodium taurocholate and sodium taurodeoxycholate), cationic (hexadecyltrimethylammonium bromide), and nonionic (Triton X-100, Triton CF-54, Tween 80, Tween 20, Cutscum and octylglucoside), were tested for their ability to activate NIL-8 sialyltransferase. The results (Figures 11 and 12) show that Triton CF-54 at 150 µg per 50 µl assay mixture (0.3 percent w/v) gave the greatest activation of the transferase. Moderate activation was observed with Triton X-100 and Cutscum, while little if any activation was noted with Tween 80, Tween 20 and octylglucoside. Both anionic and cationic detergents inactivated the enzyme. Triton CF-54, Triton X-100 and Cutscum had biphasic effects on enzyme activity; increased enzyme activity was observed at low detergent concentrations, while reduced activity was seen at higher concentrations.

As shown in Figure 13, the sialyltransferase activity had a broad pH optimum from 5.0 to 8.0. Of several buffers tested, the highest activity was observed with cacodylate-HCl at pH 6.5. The use of different buffers at the same pH did not significantly alter enzyme activity. Other glycosyltransferases have been found to have a broad pH range, with optimal activity around pH 6.0 [171,173].

Glycoconjugates which could potentially serve as sialyltransferase inhibitors were examined in transferase assays (Figure 14). Ganglioside G_{M3}, the enzyme reaction product, maximally inhibited

Figure 11 and 12. Effect of detergents on NIL-8 cell sialyltransferase activity. Varying concentrations of detergents were added to reaction mixtures and the transferase activity determined. Different cell preparations were used for experiments a and b.


Triton CF-54
Triton X-100
Cutscum
Tween 80
or
Tween 20
Product formed (pmol·mg protein⁻¹·h⁻¹)
100
75
50
25
0
50
100
150
200
Detergent Concentration (µg/50 µl assay)

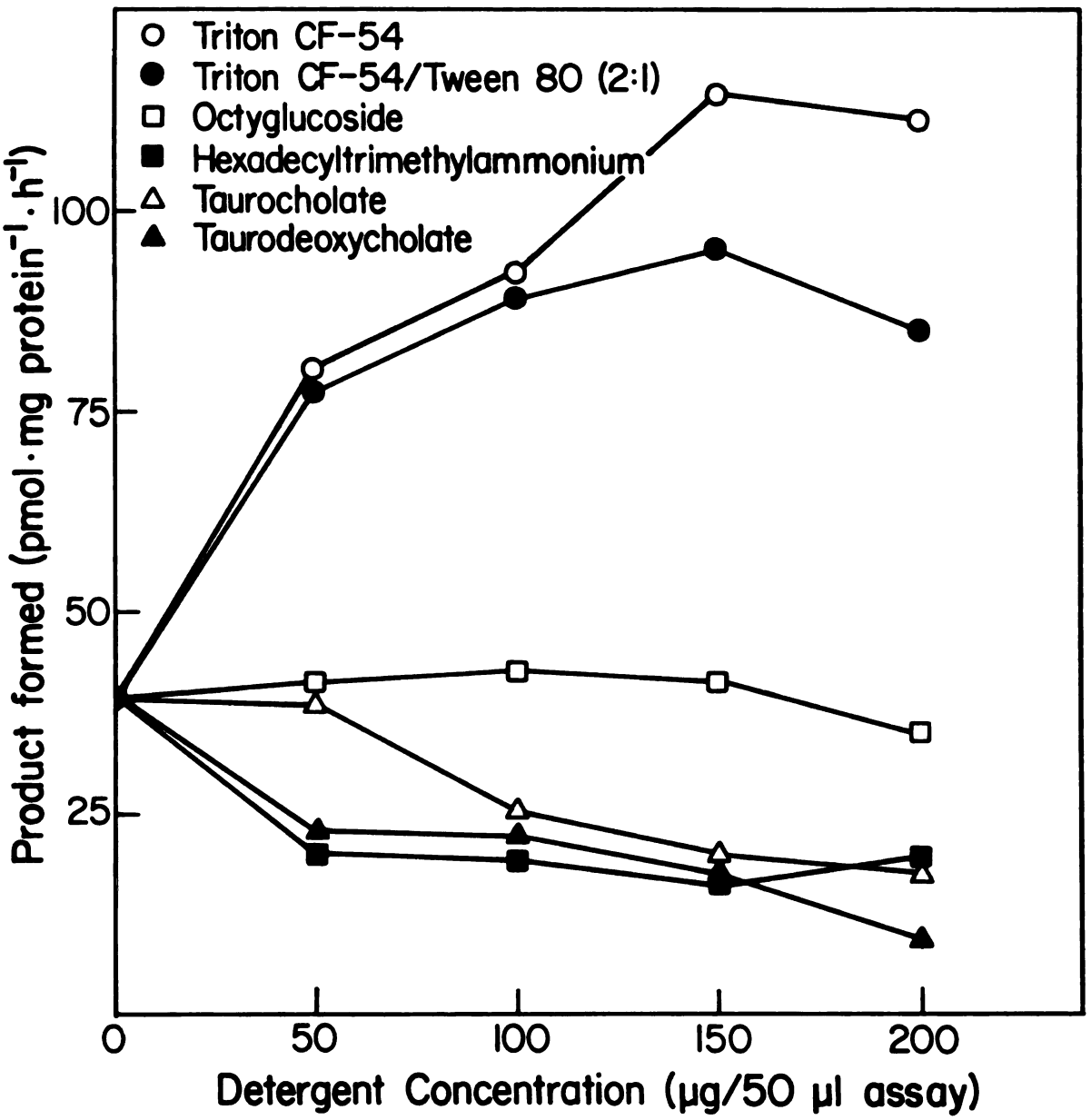

Triton CF-54
Triton CF-54/Tween 80 (2:1)
Octyglucoside
Hexadecyltrimethylammonium
Taurocholate
Taurodeoxycholate
Product formed (pmol·mg protein⁻¹·h⁻¹)
100
75
50
25
0
50
100
150
200
Detergent Concentration (µg/50 µl assay)

Figure 13. Effect of different buffers and pH on NIL-8 cell sialyltransferase activity. NaAc is sodium acetate, Mes is 2-(N-morpholino) ethanesulfonic acid, Cacodylate is dimethylarsinic acid, Tris is tris-(hydroxymethyl)amino methane, and Hepes is 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Indicated buffers were made up at various pH values within their respective buffering ranges and the transferase activity determined.

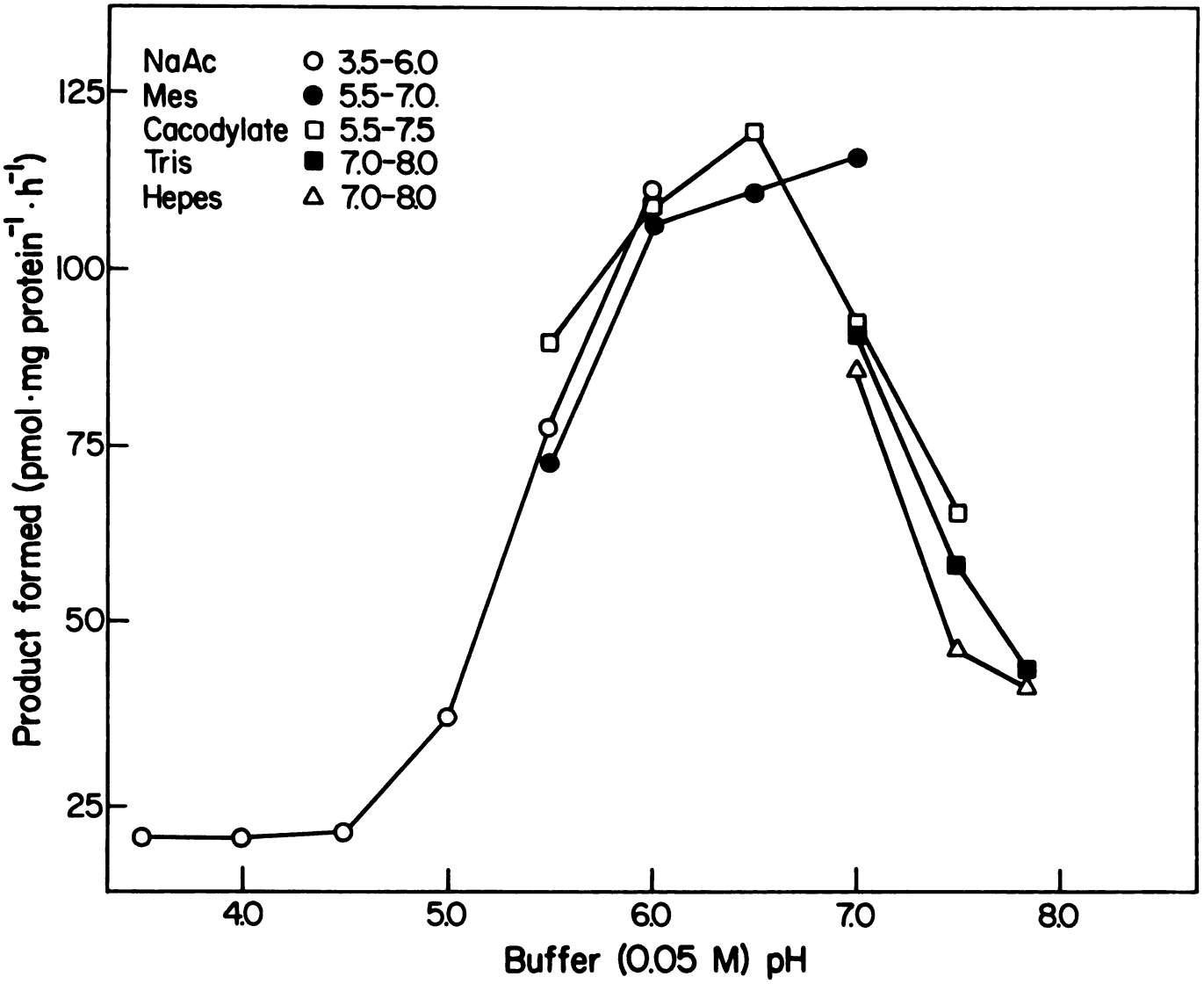
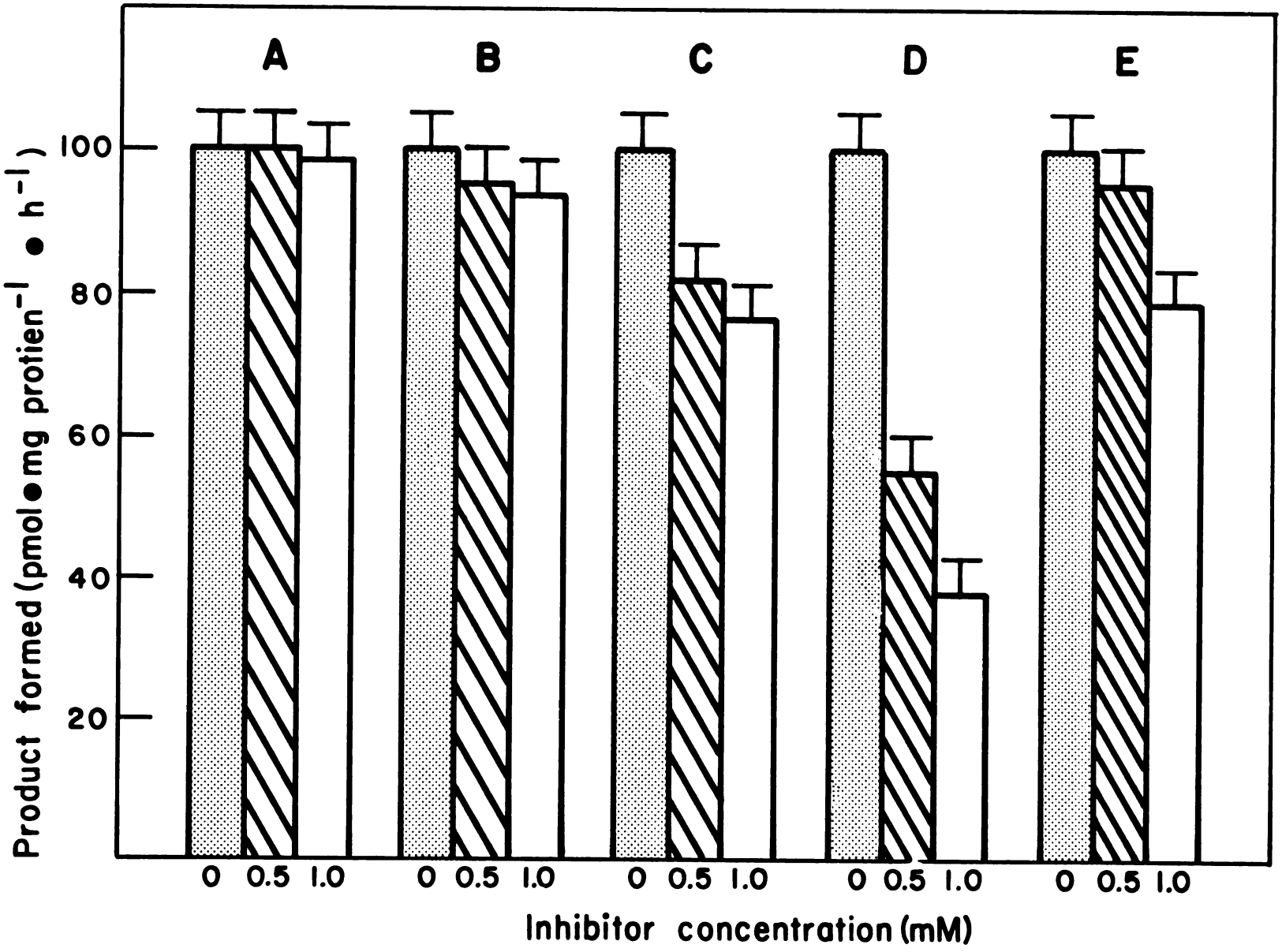

NaAc
Mes
Cacodylate
Tris
Hepes
3.5-6.0
5.5-7.0
5.5-7.5
7.0-8.0
7.0-8.0
125
100
75
50
25
Product formed (pmol·mg protein^{-1}·h^{-1})
4.0
5.0
6.0
7.0
8.0
Buffer (0.05 M) pH

Figure 14. Inhibition of CMP-sialic acid:lactosylceramide sialyltransferase by potential glycoconjugate inhibitors. Indicated amounts of glucosylceramide (A), globotriaosylceramide (B), globotetraosylceramide (C), G_{M3} ganglioside (D) and desialized fetuin (E).


A
B
C
D
E
100
80
60
40
20
Product formed (pmol • mg protien^{-1} • h^{-1})
0 0.5 1.0
0 0.5 1.0
0 0.5 1.0
0 0.5 1.0
0 0.5 1.0
Inhibitor concentration (mM)

sialyltransferase activity, while glucosylceramide, globotriaosylceramide, globotetraosylceramide, and desialized fetuin inhibited activity to a lesser extent.

The NIL-8 cell cycle was characterized by ^{3}H-thymidine incorporation to define S phase, cell number determination and mitotic figure staining to define M phase, Figure 15. ^{3}H-Thymidine incorporation reached a peak 12 hours after the the plating of the synchronized cells. Subsequently, the cells began dividing rapidly and the cell count almost doubled during the period between 17-24 hours after plating. During this period of time, M/early G phase, the percent of cells in mitosis reached a maximum. During this period of time the cells were distributed in M and early G_1 phase. The second peak of ^{3}H-thymidine incorporation occurred 34 hours after the plating of the synchronized cells; cell counts remained almost unchanged during both S phases examined.

Table 18 contains the specific activities of CMP-sialic acid:lactosylceramide sialyltransferase and α-galactosidase A at different phases of the cell cycle. The lysosomal hydrolase, α-galactosidase A, was examined since it was previously observed that the specific activities of glycosylhydrolases were highest during late S and early G_2 phases of the cell cycle [174]. CMP-sialic acid:lactosylceramide sialyltransferase exhibited activity throughout the cell cycle; however, peak activities were found in M/early G and G_1 phases. Cells in G_2 and S phases were found to have lower sialyltransferase activity. Regulation of sialyltransferase activity during the cell growth cycle was observed. In agreement with previous results, α-galactosidase A activity was found to be highest during the S and G_2 phases

Figure 15. Characterization of NIL-8 cell cycle. Three different methods were used to characterize the cycle of NIL-8 cells synchronized by a combination of double thymidine block with colcemid treatment to collect mitotic cells as described in Methods.

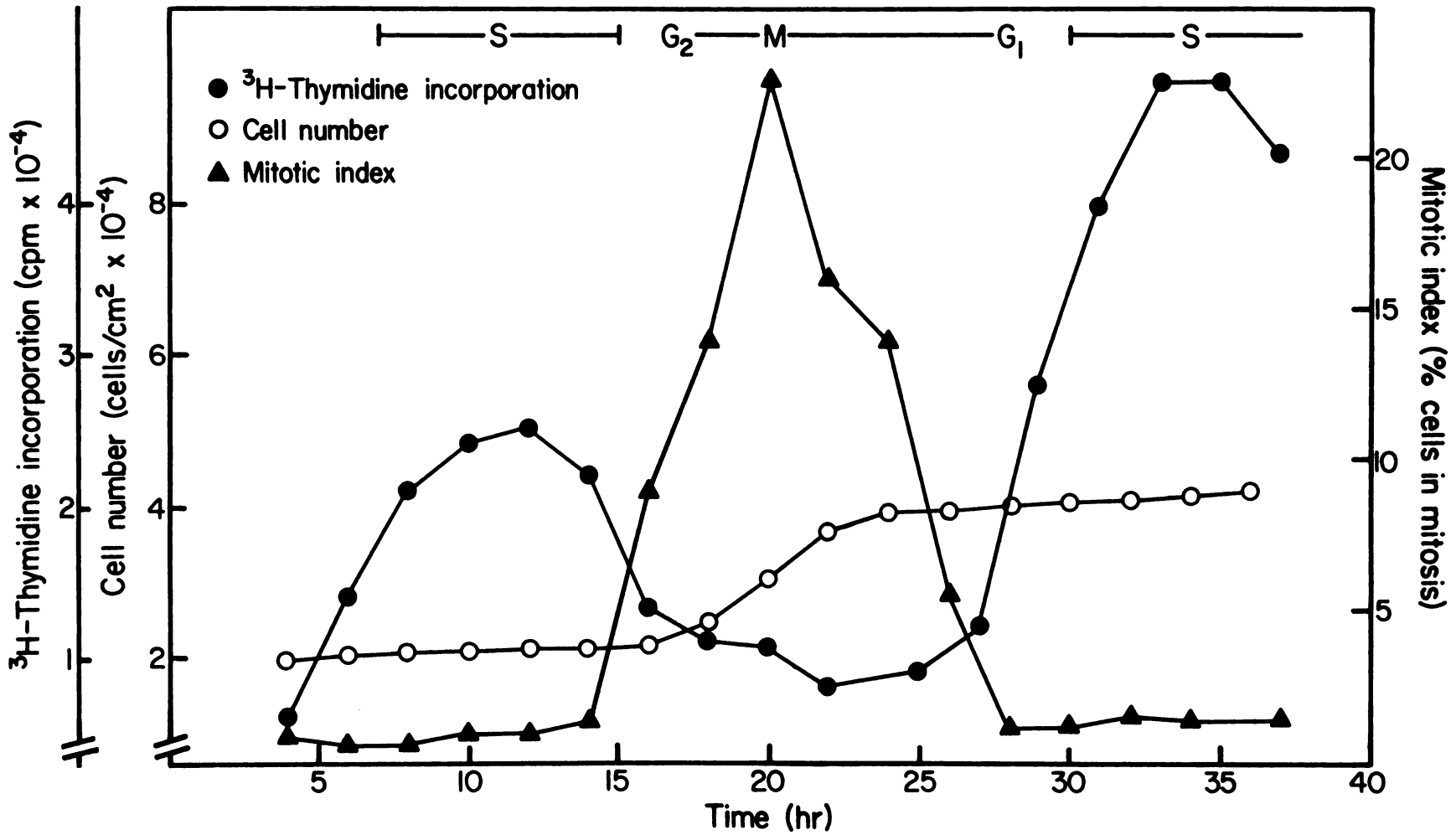
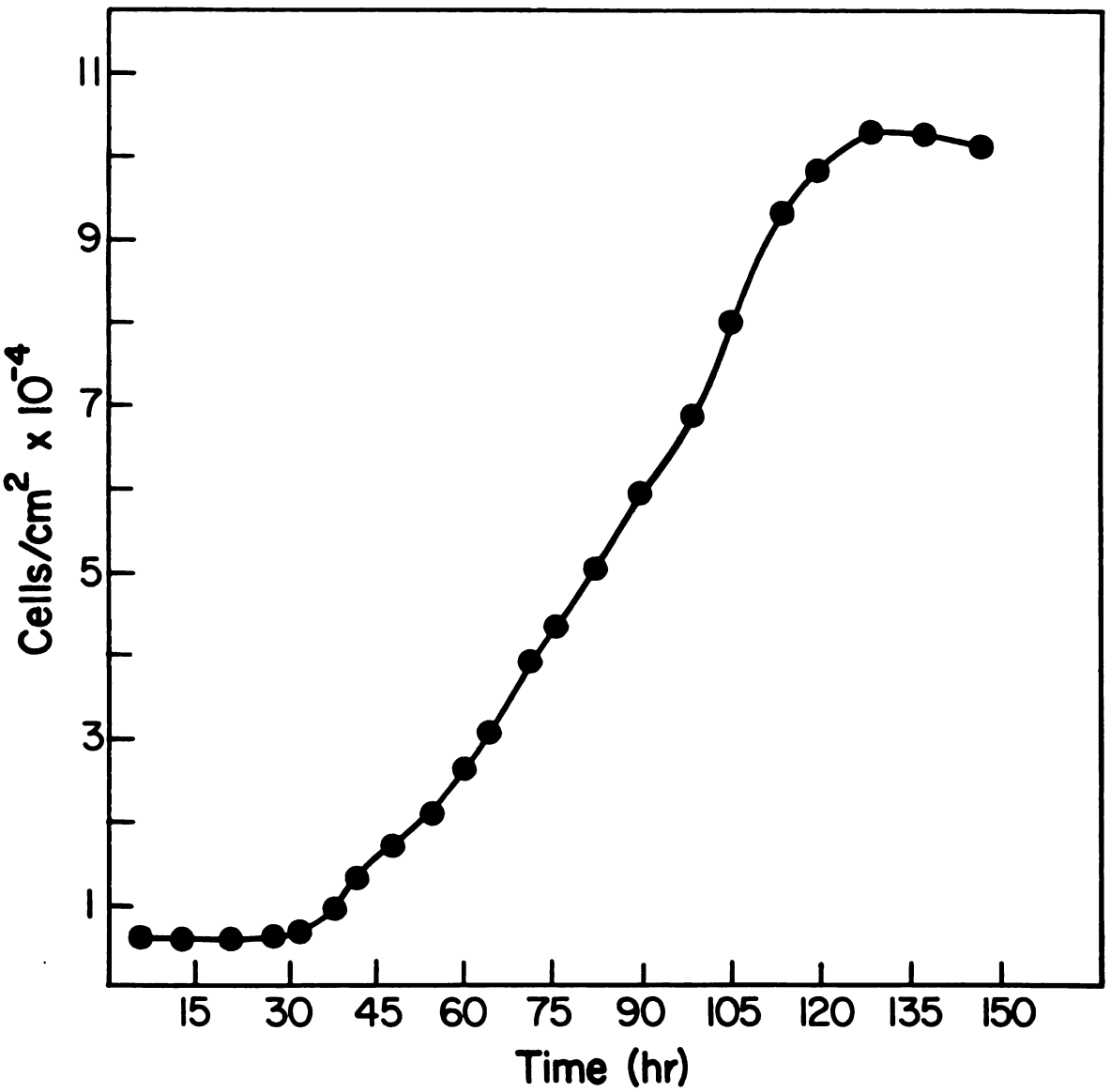

S
G2
M
G1
S
● 3H-Thymidine incorporation
○ Cell number
▲ Mitotic index
3H-Thymidine incorporation (cpm x 10-4)
Cell number (cells/cm2 x 10-4)
Mitotic index (% cells in mitosis)
Time (hr)
1
2
3
4
2
4
6
8
5
10
15
20
5
10
15
20
25
30
35
40

Table 18. Effect of NIL-8 cell cycle on CMP-sialic acid:lactosylceramide sialyltransferase and α-galactosidase A activity.[a]

Time (hours)	Sialytransferase (pmol/mg protein/h)	α-Galactosidase A (nmol/mg protein/h)
12 (S phase)	74 ± 3	491 ± 19
16 (G_2 phase)	94 ± 3	508 ± 20
20 (M/early G_1 phase)	205 ± 16	279 ± 22
26 (G_1 phase)	138 ± 11	309 ± 12

[a] Cells were synchronized as described in Methods.

Figure 16. NIL-8 cell growth characteristics as determined by total cell number. Estimations of cell growth were made in triplicate approximately every six hours.


Cells/cm² x 10⁻⁴
1
3
5
7
9
11
15
30
45
60
75
90
105
120
135
150
Time (hr)

[150].

When the growth characteristics of NIL-8 cells were determined (Figure 16), a final saturation density of 1.1 x 10^5 cells/cm^2 and a 29 hour doubling time were observed. The specific activity of CMP-sialic acid:lactosylceramide sialyltransferase differed between exponential cell growth and density-dependent inhibition of cell growth. The sialyltransferase activity increased as the cells reached density-dependent growth inhibition, from 121 pmol/mg protein/hr in sparsely growing cells (5 x 10^4 cells/cm^2) to 237 pmol/mg protein/hr in confluent cells (10^5 cells/cm^2).

IV. Effect of Phorbol Esters on CMP-Sialic Acid:Lactosylceramide Sialyltransferase in Various Systems.

Phorbol treatment of V79 cells resulted in increased CMP-sialic acid:lactosylceramide sialyltransferase activity, Table 19. Table 20 shows the results of PMA on sialyltransferase activity in KB, CHO and V79 cells. These cell lines also showed an elevation in sialyltransferase activity. The effect of PMA treatment with time on sialyltransferase activity in V79 cells is presented in Figure 17. Enzyme activity began to increase after eight hours of PMA treatment, reached a maximum relative to controls at approximately twenty hours, and declined after thirty-six hours of PMA treatment. It appears from these fingings that increased sialyltransferase activity may in part account for the mechanism by which cellular gangliosides, particularly G_{M3}, increase in V79 cells treated with tumor- promoting phorbol esters.

Table 19. The effect of phorbol esters on V79 cell CMP-sialic acid:lactosylceramide sialytransferase activity.

Treatment	Sialyltransferase activity[a]	
	pmoles/mg protein/h	Percent increase
Control	198 ± 10	-
4α-PDD	200 ± 5	-
PDB	242 ± 7	22
PMA	329 ± 6	66

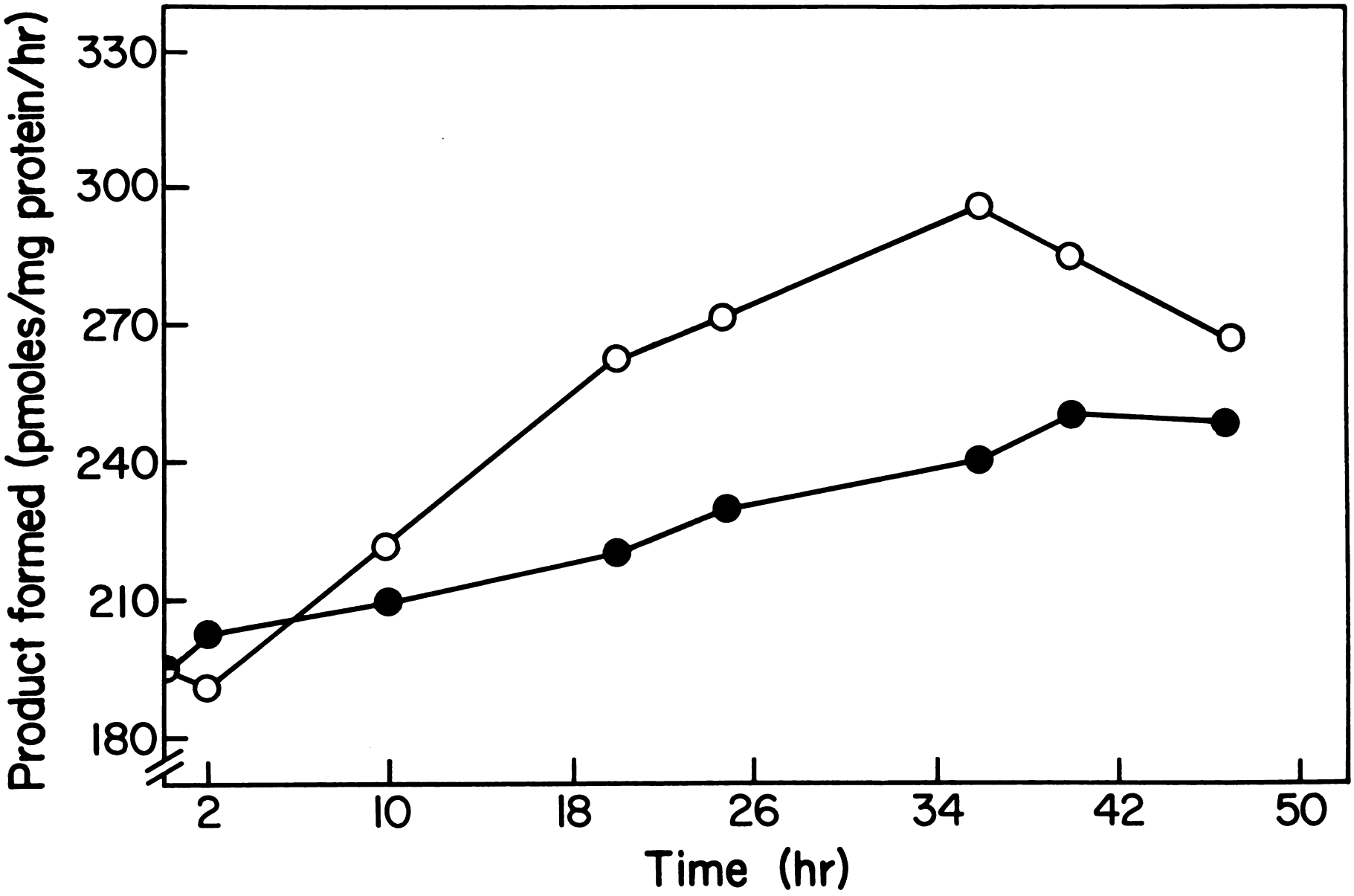
[a] Values represent the mean of three determinations. Standard deviations were less than 10% of the mean value. 4a-PDD, 4a-phorbol-12,13-didecanoate; PBS, phorbol-12,13-dibutyrate; PMA, phorbol-12-myristate-13-acetate.

Table 20. The effect of PMA on CMP-sialic acid:lactosylceramide sialyltransferase activity in V79, KB and CHO cell lines.

Cell line	Control[a] pmoles/mg protein/hour	PMA treated[a] pmoles/mg protein/hour
V79	200	325
KB	477	2605
CHO	47	306

[a] Values represent the mean of three experiments.

Figure 17. PMA effect with time on V79 cell CMP-sialic acid:lactosylceramide sialyltransferse activity. O, PMA treatment was 1.6 μM; while ●, control contained no drug.


Product formed (pmoles/mg protein/hr)
330
300
270
240
210
180
2
10
18
26
34
42
50
Time (hr)

V. Partial Purification of CMP-Sialic Acid:Lactosylceramide Sialyltransferase.

The specific activity of CMP-sialic acid:lactosylceramide sialyltransferase was enriched 28-fold in Golgi membrane fractions of chicken liver, which were recovered at the 0.7 to 1.2 M sucrose interface following discontinuous sucrose gradient centrifugation. The recovery of activity in this fraction was relatively quantitative (85 to 105 percent over various runs) compared to the activity in liver homogenates, Table 21.

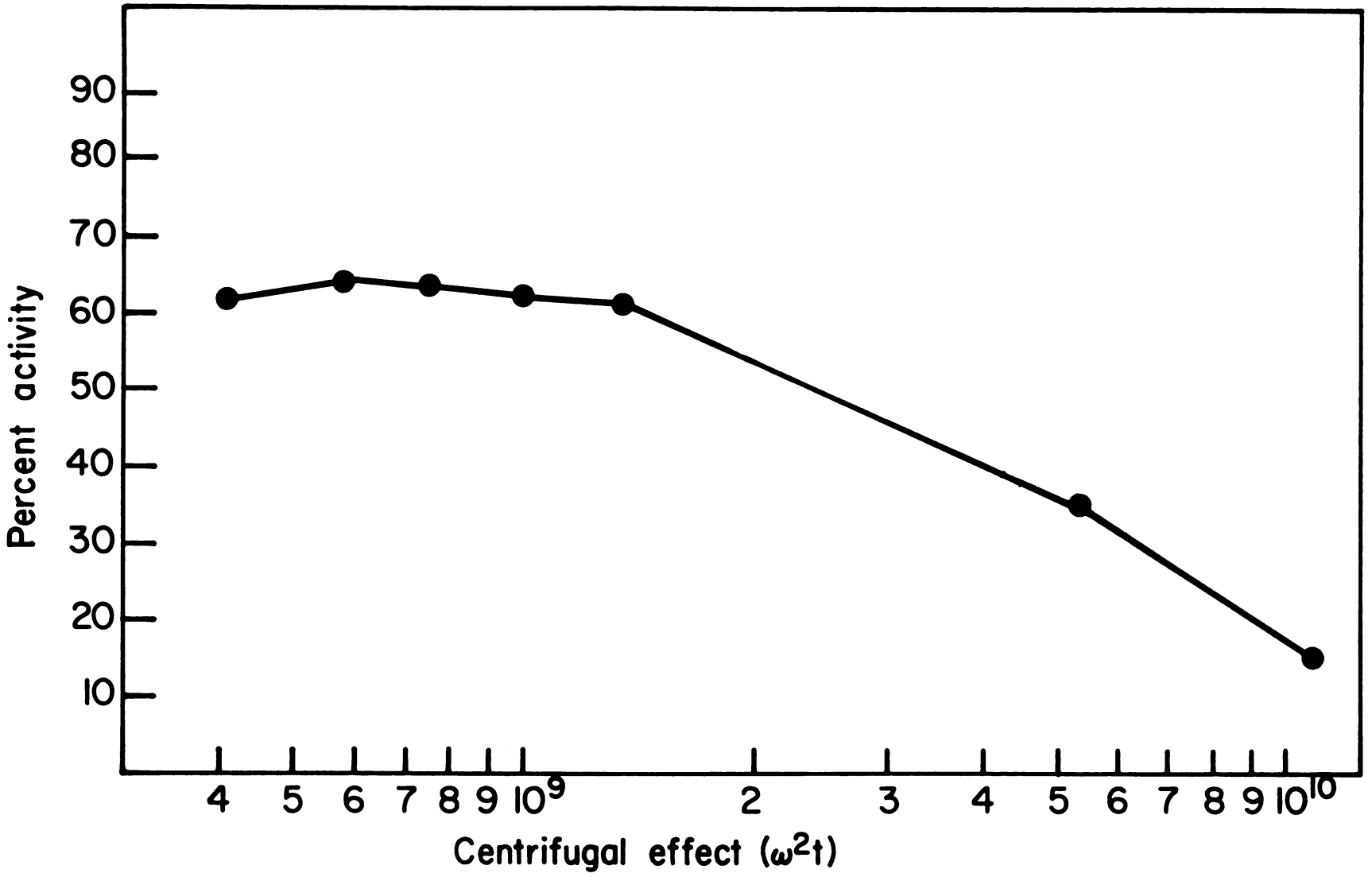
Recovery of the sialyltransferase activity in the concentration step was nearly quantitative (93 percent). Later extraction of the enzyme from Golgi membranes was found to be affected by the centrifugal force at which the membranes were pelleted, Figure 18. Centrifugation at 50,000 Xg for 15 min ($w^2t = 5.4 \times 10^9$) was used routinely for concentration of Golgi membranes.

Most glycosyltransferases are bound to the endoplasmic reticulum and Golgi apparatus, and like most other membrane-bound proteins they generally require detergent solubilization for purification. During purification they may also require detergent to prevent aggregation and inactivation [175]. Treatment of the concentrated Golgi membranes with Triton X-100 and glycerol together with sonication proved to be the most effective method for achieving release of the enzyme activity. Following sonication and detergent/glycerol treatment of the concentrated Golgi membrane fraction, the sialyltransferase activity which remained in the supernatant fraction after centrifugation at 150,000 Xg for 90 min was considered to be solubilized. Under these same conditions the CMP-sialic acid:lactosylceramide sialyltransferase activity

Table 21. Partial purification of CMP-sialic acid:lactosylceramide sialyltransferase.

Enzyme Preparation	Total units (pmoles/hour)	Total protein (mg)	Specific activity (pmoles/mg protein/hour)	Purification (-fold)	Recovery (percent)
Homogenate	40200	1680	24		100
Golgi membranes	36600	55	670	28	91
Solubilized enzyme	24000	23	1040	43	60
Cellulose-phosphate	25000	18	1390	58	62
Sephadex G-150	22500	7.4	3050	127	56

Figure 18. The effect of centrifugal force at which Golgi membranes are pelleted on subsequent extraction of CMP-sialic acid:lactosylceramide sialyltransferase.


Percent activity
90
80
70
60
50
40
30
20
10
4
5
6
7
8
9
10^9
2
3
4
5
6
7
8
9
10^10
Centrifugal effect (ω²t)

of intact Golgi membranes is quantitatively pelleted. Extraction of the membrane preparation with Triton X-100 at a concentration between 0.5 and 1.5 percent (w/v) gave maximal recovery of sialyltransferase activity in the supernate, Figure 19. In addition, the concentration of glycerol in the extraction buffer also affected the recovery of activity in the supernate, Figure 20. Triton X-100 at 0.6 percent (v/w) along with glycerol at forty percent (w/v) were used in the extraction of Golgi membranes. Solubilization of CMP-sialic acid:lactosylceramide sialyltransferase activity from Golgi membranes resulted in a 43-fold purification and a 60 percent recovery of activity.

The solubilized enzyme preparation was applied to a cellulose-phosphate column and eluted with extraction buffer, Figure 21. Recovery of enzyme activity was quantitative and the total purification was 58-fold, Table 21. Pooled fractions from ion exchange chromatography were applied to a Sephadex G-150 column, Figure 22. The sialyltransferase activity chromatographed as a broad peak with the bulk of the recovered protein coinciding with the peak of enzyme activity, thus resulting in approximately two-fold purification (127-fold overall). Recovery of the enzyme activity was again nearly quantitative compared to previous steps, Table 21.

VI. The Effect of Phospholipids on Partially Purified CMP-Sialic Acid:Lactosylceramide Sialyltransferase Activity.

Various phospholipids were tested for their ability to modulate sialyltransferase activity *in vitro*. The results (Figures 23 and 24) indicate that phosphatidylcholine gave the greatest activation of the

Figure 19. The effect of Triton X-100 concentration on the solubilization of CMP-sialic acid:lactosylceramide sialyltransferase activity from Golgi membranes.

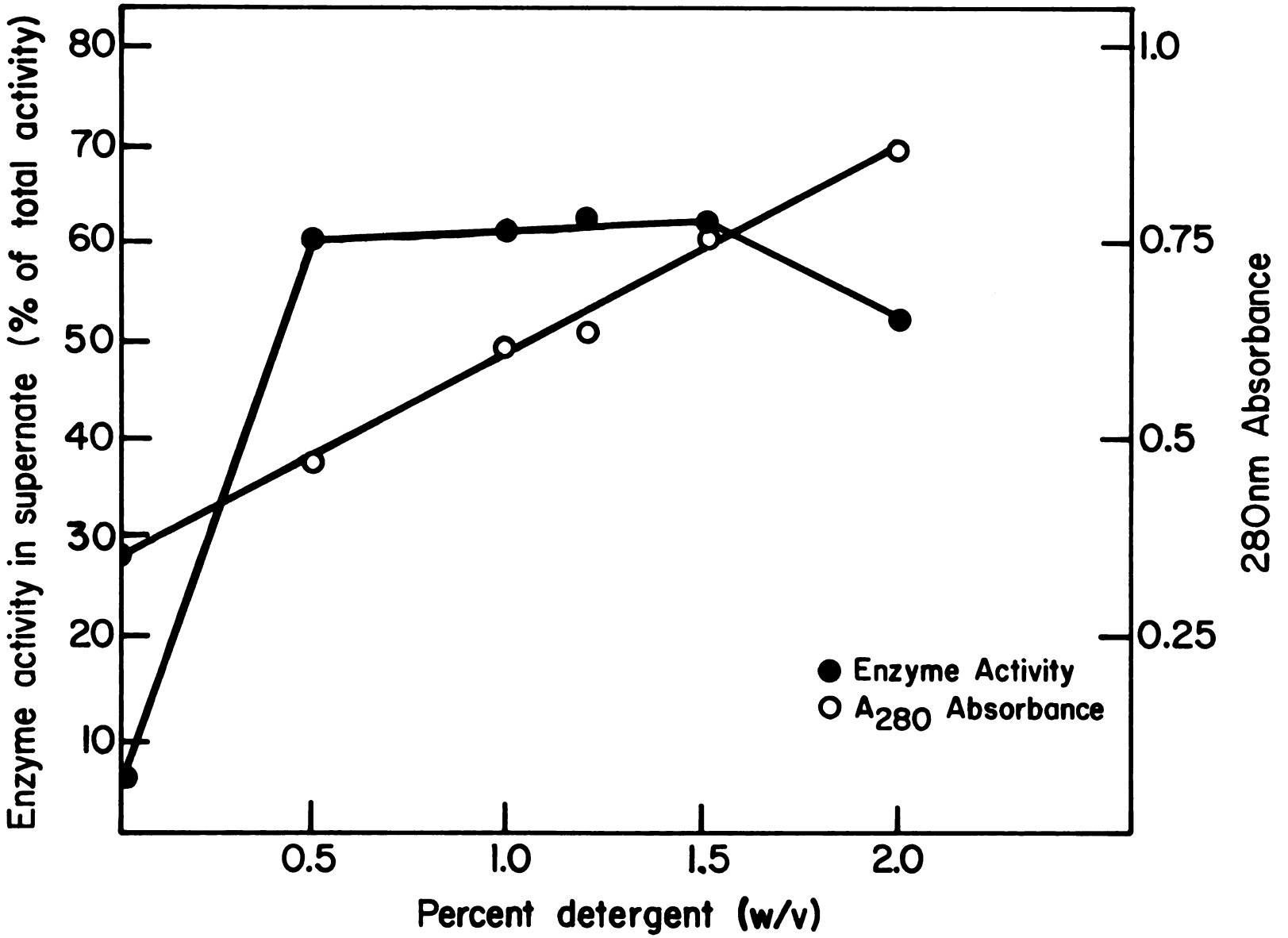

Enzyme activity in supernate (% of total activity)
80
70
60
50
40
30
20
10
1.0
0.75
0.5
0.25
280nm Absorbance
0.5
1.0
1.5
2.0
Percent detergent (w/v)
Enzyme Activity
A280 Absorbance

Figure 20. The effect of glycerol concentration on the solubilization of CMP-sialic acid:lactosylceramide sialyltransferase activity from Golgi membranes.

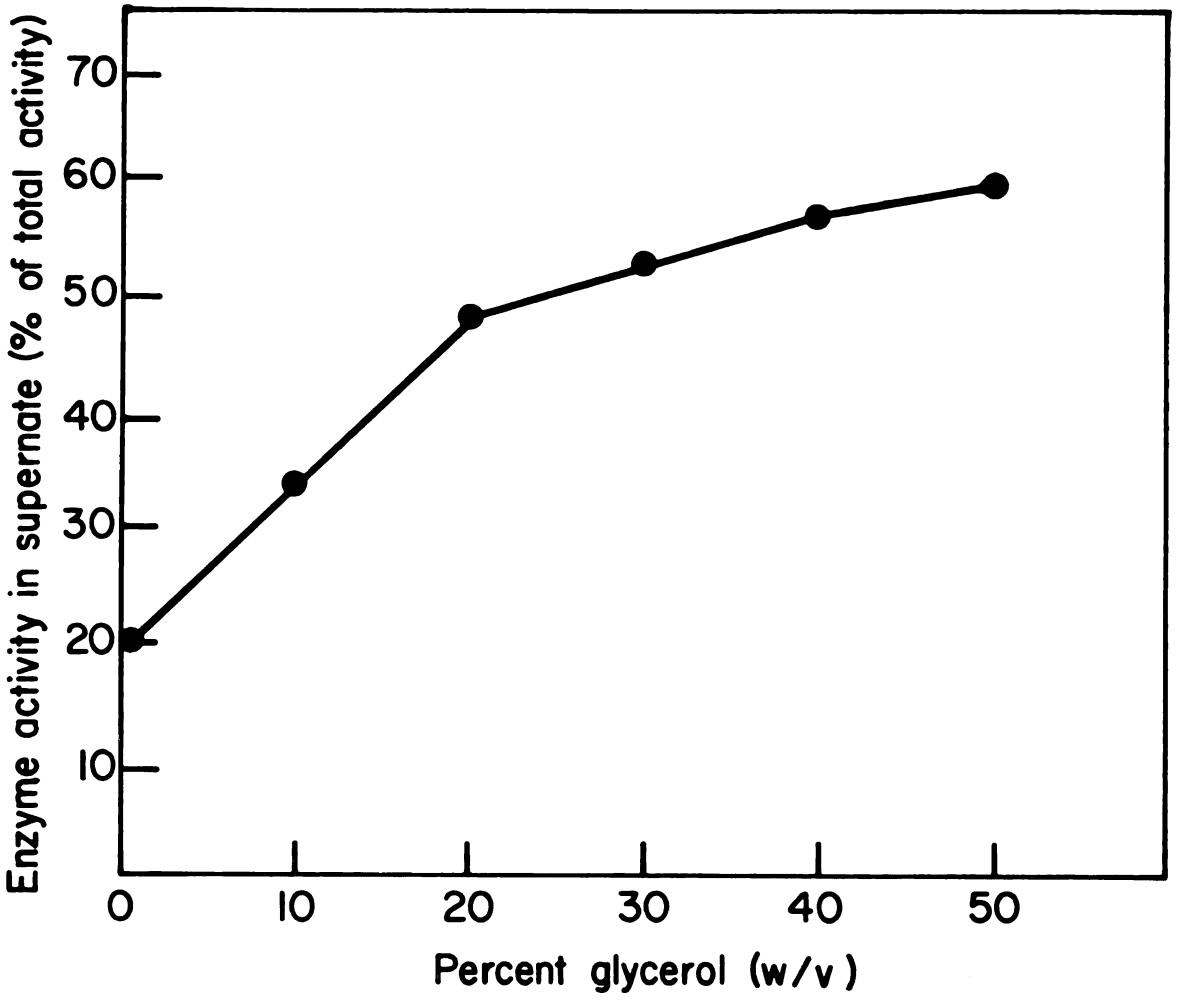

Enzyme activity in supernate (% of total activity)
70
60
50
40
30
20
10
0
10
20
30
40
50
Percent glycerol (w/v)

Figure 21. The elution profile of CMP-sialic acid:lactosylceramide sialyltransferase activity from a cellulose-phosphate chromatographic column, as described in Methods.

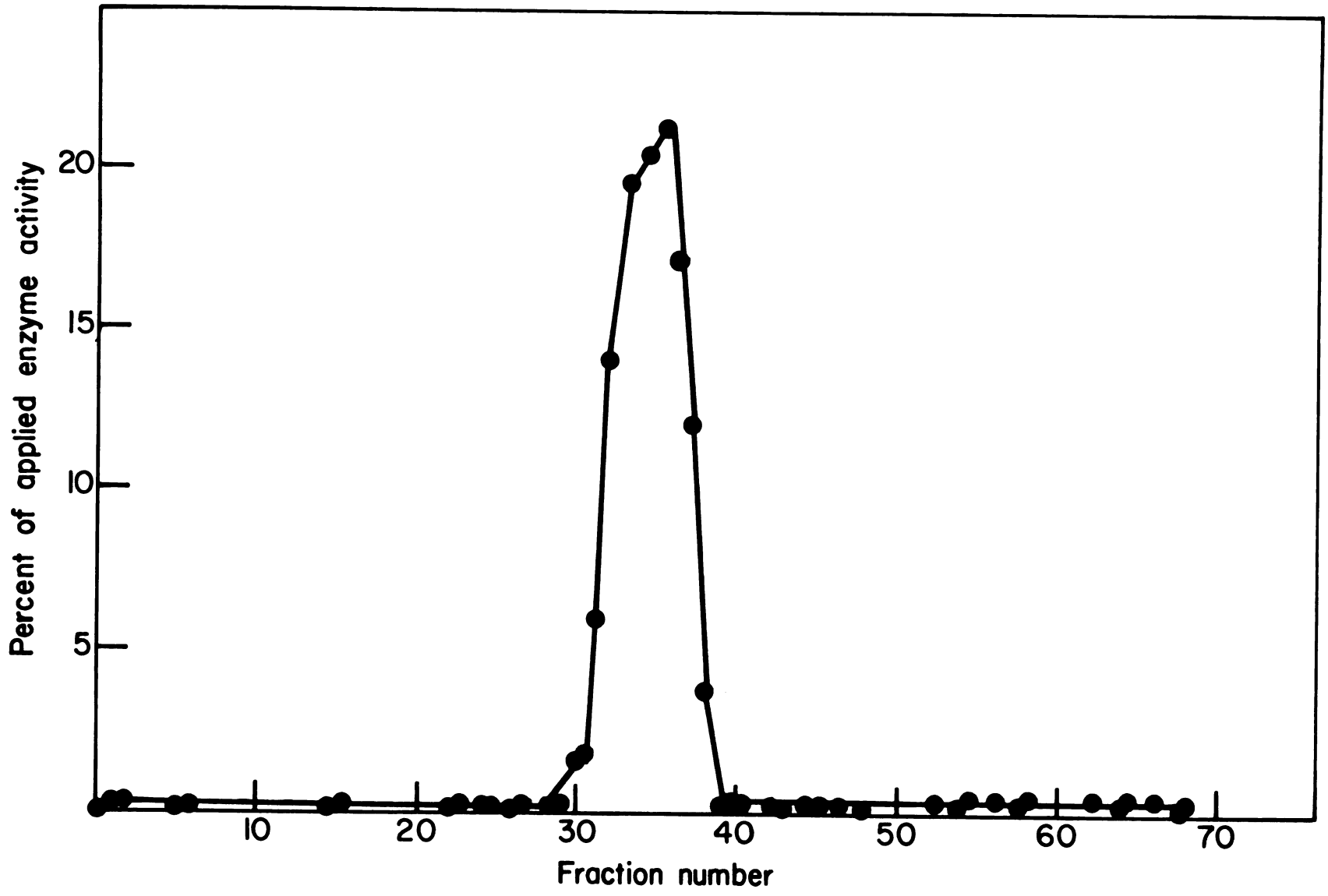

Percent of applied enzyme activity
20
15
10
5
10
20
30
40
50
60
70
Fraction number

Figure 22. The elution profile of CMP-sialic acid:lactosylceramide sialyltransferase activity through a Sephadex G-150 chromatographic column, as described in Methods.

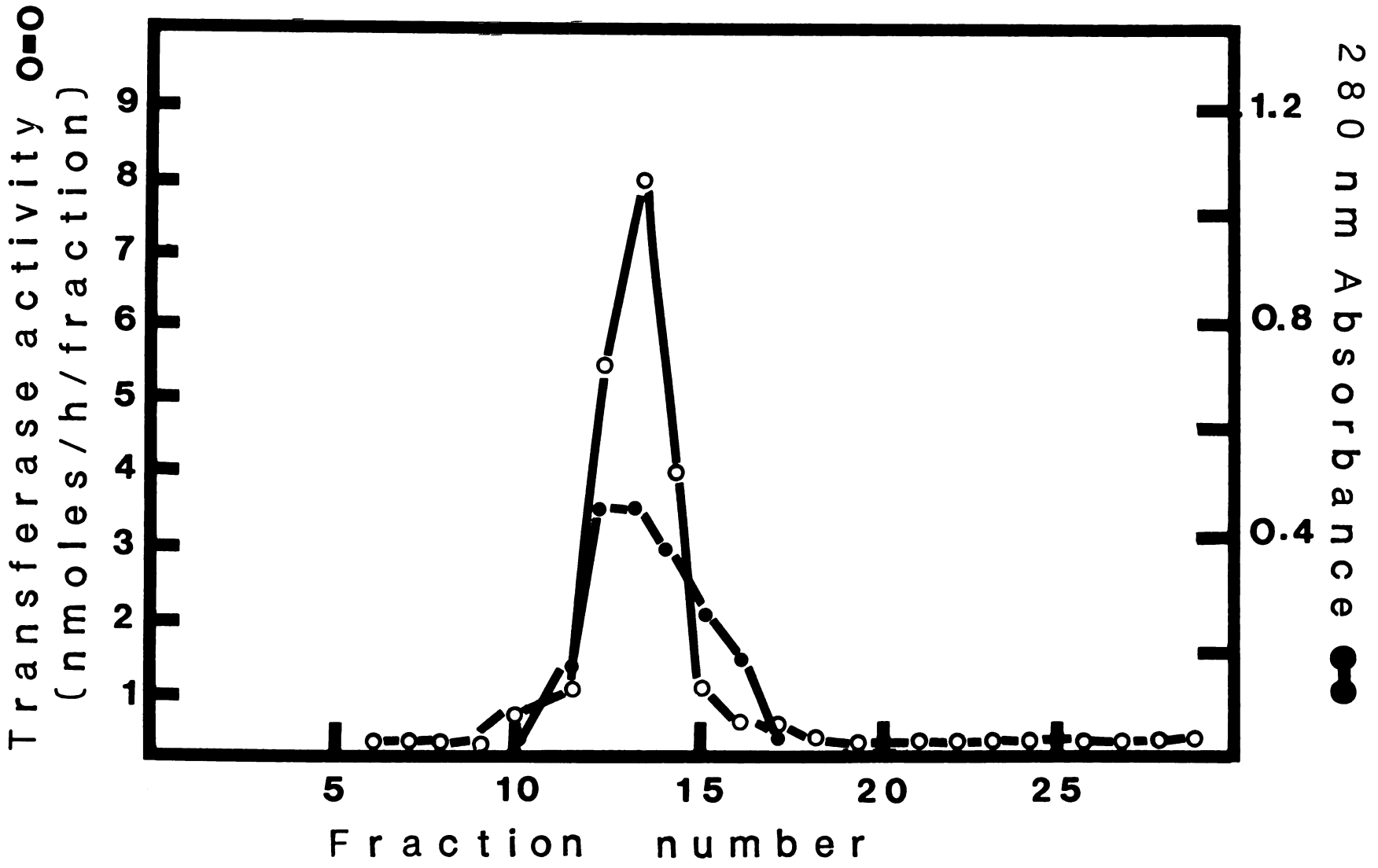

Transferase activity O=O
(nmoles/h/fraction)
9
8
7
6
5
4
3
2
1
1.2
0.8
0.4
280 nm Absorbance
5
10
15
20
25
Fraction number

Figure 23. The effect of phospholipids on CMP-sialic acid:lactosylceramide sialyltransferse activity. PC is phosphatidylcholine; PE is phosphatidylethanolamine; PA is phosphatidic acid; PI is phosphatidylinositol; and PS is phosphatidylserine.

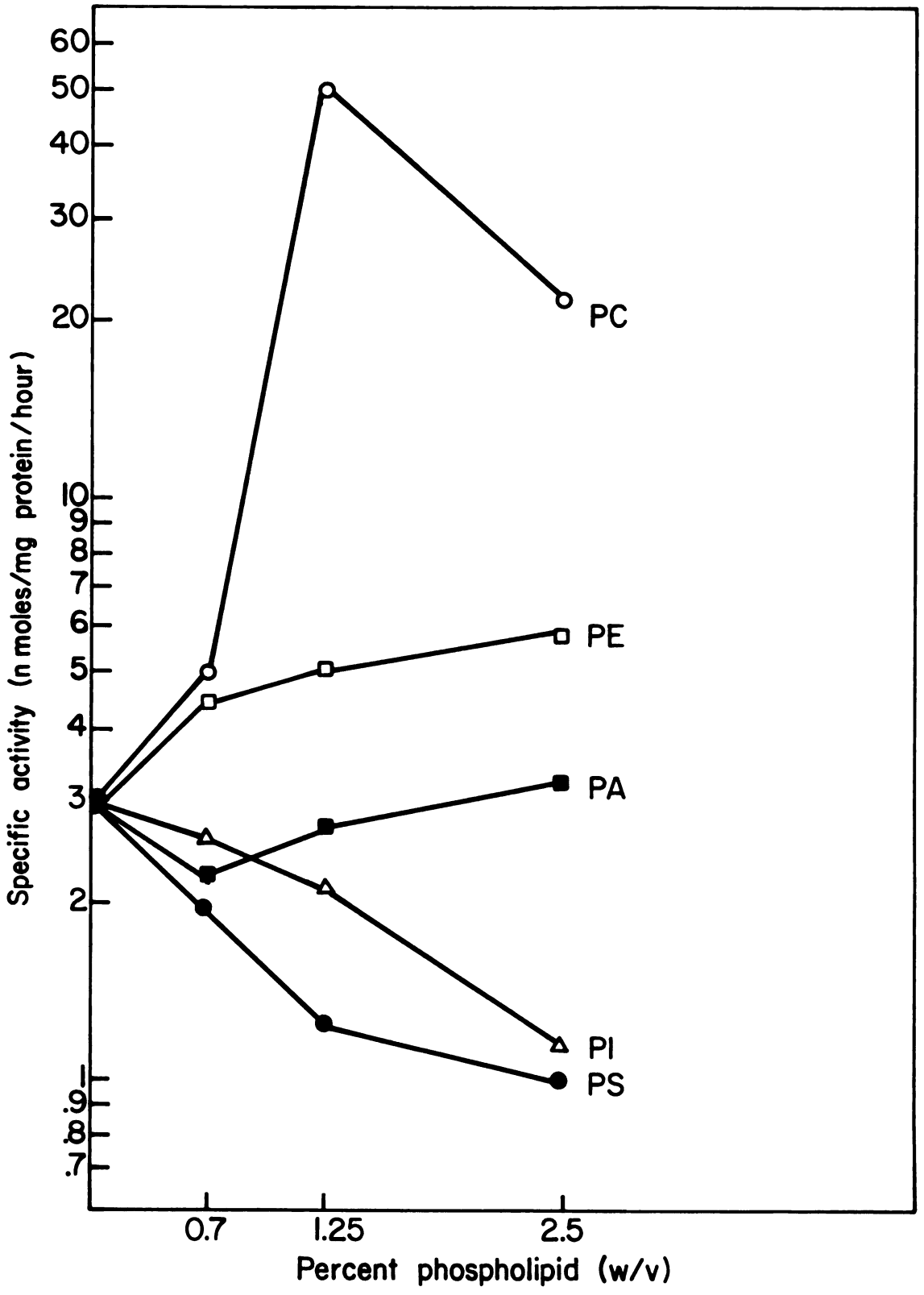
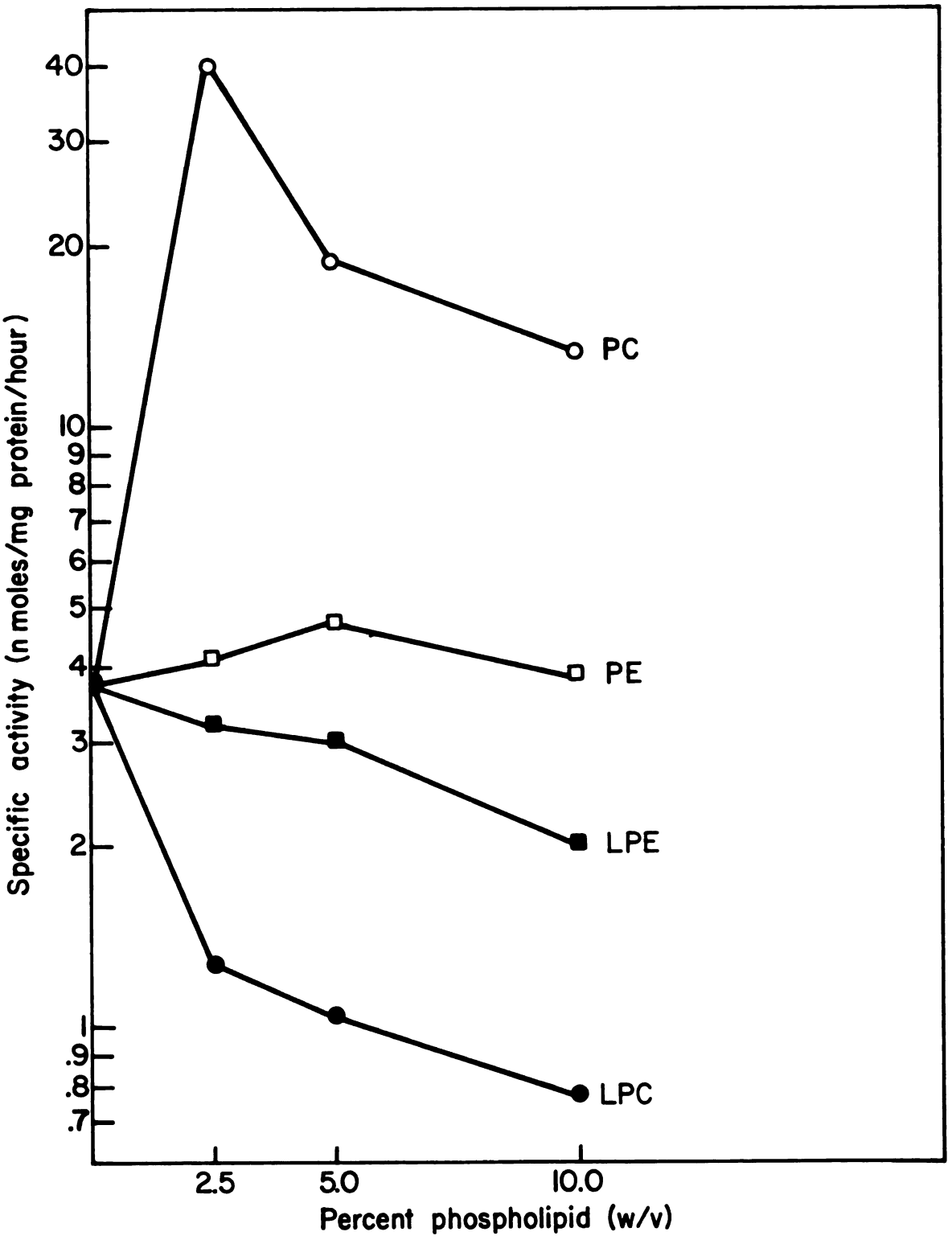

60
50
40
30
20
10
9
8
7
6
5
4
3
2
1
.9
.8
.7
Specific activity (n moles/mg protein/hour)
PC
PE
PA
PI
PS
0.7
1.25
2.5
Percent phospholipid (w/v)

Figure 24. The effect of diacylphospholipids and lysophospholipids on CMP-sialic acid:lactosylceramide sialyltransferase activity. PC is phosphatidylcholine; PE is phosphatidylethanolamine; LPC is lysophosphatidylcholine; and LPE is lysophosphatidylethanolamine.


40
30
20
10
9
8
7
6
5
4
3
2
.9
.8
.7
Specific activity (n moles/mg protein/hour)
PC
PE
LPE
LPC
2.5
5.0
10.0
Percent phospholipid (w/v)

transferase. Moderate activation was observed with phosphatidylethanolamine, while little effect was noted with phosphatidic acid. Phosphatidylcholine and phosphatidylethanolamine had biphasic effects on enzyme activity; increased enzyme activity was observed at lower phospholipid concentrations, while reduced activity was seen at higher concentrations. Both phosphatidylinositol and phosphatidylserine inactivated the enzyme, as did lysophosphatidylcholine and lysophosphatidylethanolamine. Of the monoacylphospholipids, lysophosphatidylcholine gave the greatest inactivation, while lysophosphatidylethanolamine moderately inactivated the enzyme.

VII. Phosphorylation-Dephosphorylation of CMP-Sialic Acid:Lactosylceramide Sialyltransferase

When the partially purified enzyme was incubated for one hour with increasing amounts of bovine intestinal alkaline phosphatase, CMP-sialic:lactosylceramide sialyltransferase activity decreased and eventually reached a plateau, Figure 25. Moreover, incubation of the partially purified enzyme for increasing amounts of time with the 50 µg/ml of bovine intestinal alkaline phosphatase also resulted in decreased sialyltransferase activity, Figure 26. This experiment eliminates the possibility that bovine alkaline phosphatase or a component in the preparation is an inhibitor of sialyltransferase activity or is an alternate acceptor of sialic acid via the sialyltransferase. Since incubations of sialyltransferase with the phosphatase for various periods of time occured before reactions with substrate, the same amount of the phosphatase was present in reaction mixtures during determination of enzyme activity. Addition of phosphate to the phosphatase incubations

Figure 25. The effect of bovine intestinal alkaline phosphatase concentration on the partially purified CMP-sialic acid:lactosylceramide sialyltransferase activity.

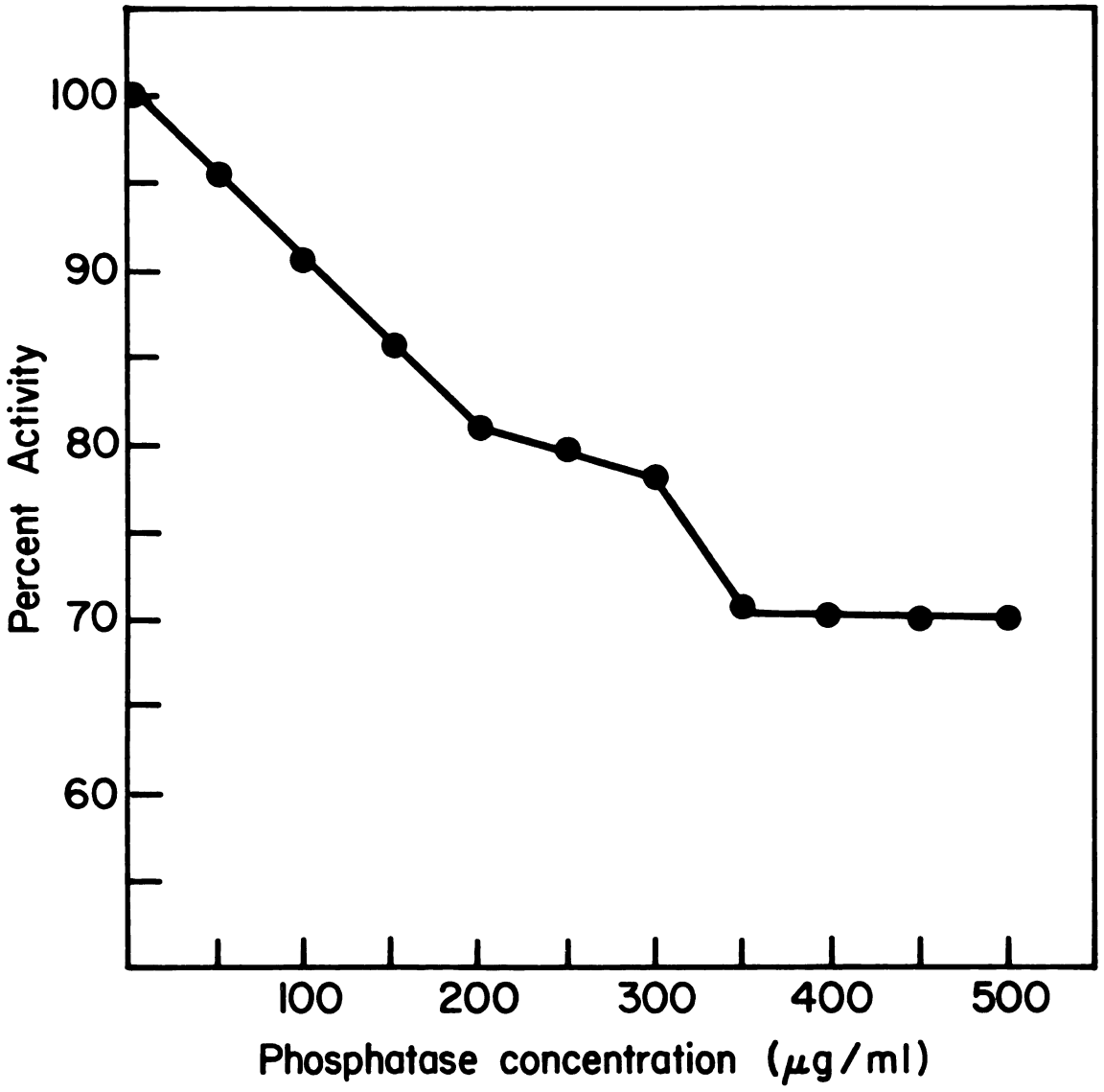

100
90
80
70
60
Percent Activity
100
200
300
400
500
Phosphatase concentration (μg/ml)

Figure 26. The effect of bovine intestinal alkaline phosphatase treatment with time on the partially purified CMP-sialic acid:lactosylceramide sialyltransferase activity.

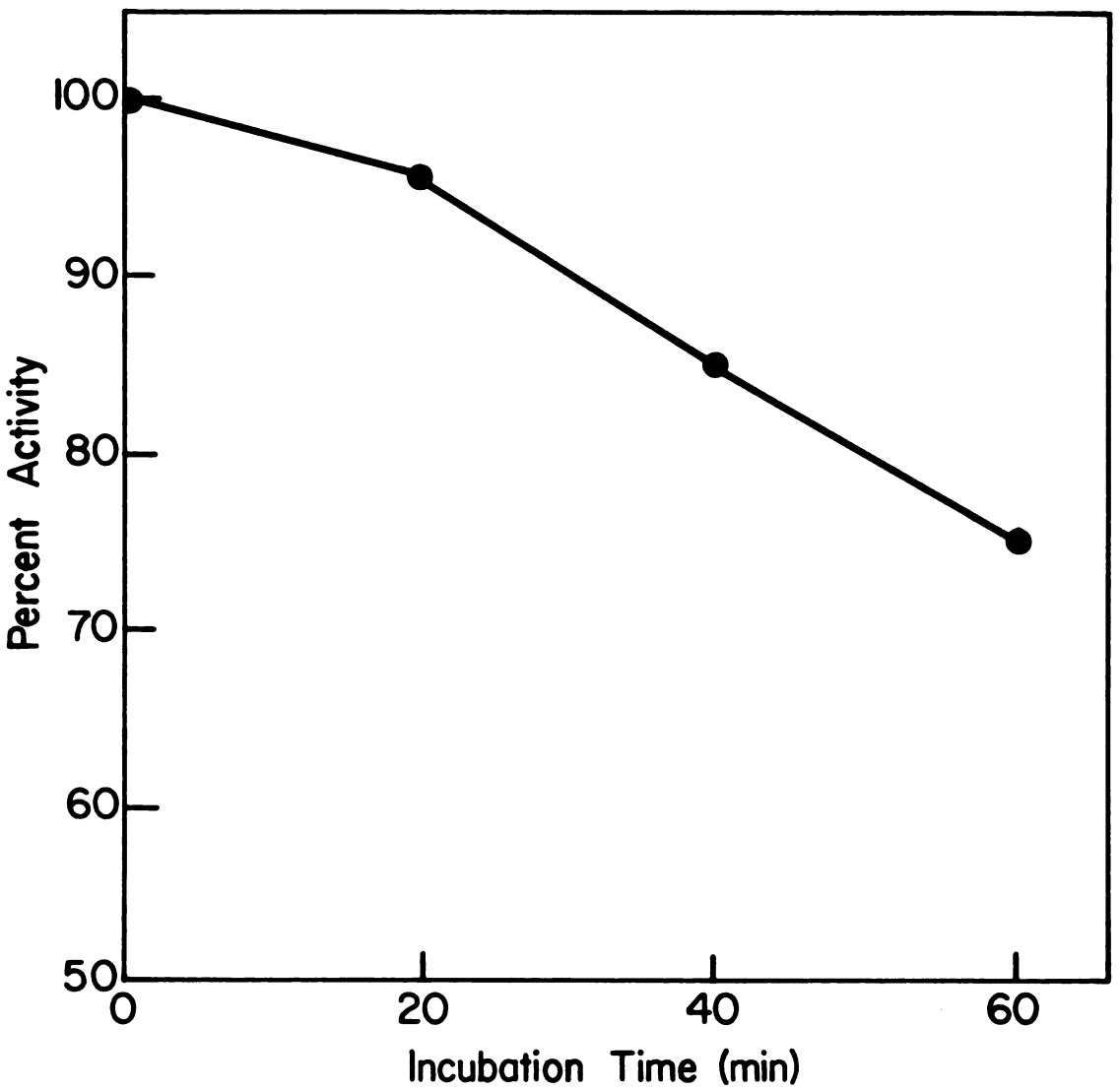

100
90
80
70
60
50
Percent Activity
0
20
40
60
Incubation Time (min)

Table 22. The effect of phosphatase on CMP-sialic acid:lactosylceramide sialyltransferse activity.

Preparation	Percent of Control
Control	100
Bovine intestine alkaline phosphatase	54
Bovine intestine alkaline phosphatase plus sodium fluoride	70
Bovine intestine alkaline phosphatase plus sodium phosphate	113
Boiled bovine intestine alkaline phosphatase	98
Wheat germ acid phosphatase	100
Bacterial alkaline phosphatase	96

completely prevented the reduction of sialyltransferase activity by the phosphatase, while fluoride only slightly prevented the reduction, Table 22. Phosphate at 50 mM concentration was found to inhibit bovine intestinal alkaline phosphatase under the same conditions used to phosphatase treat the partially purified sialyltransferase, while fluoride had no effect on the phosphatase, Figure 27. These conditions also resulted in a diminished bovine intestinal alkaline phosphatase specific activity with an artificial substrate (1.2 μmoles of PNP-phosphate hydrolyzed/ min/mg protein) when compared to the specific activity under optimal conditions (4.0 μmoles of PNP-phosphate hydrolyzed/min/mg protein). Both bacterial alkaline phosphatase and wheat germ acid phosphatase were found to be ineffective in reducing sialyltransferase activity. Incubation of bovine intestinal alkaline phosphatase with CMP-sialic acid, lactosylceramide or ganglioside G_{M3} did not result in their degradation as determined by thin-layer chromatography. This eliminates phosphatase degradation of substrate or product resulting in less net product formation as a possible artifact.

Incubation of partially purified CMP-sialic acid:lactosylceramide sialyltransferase under phosphorylating conditions resulted in enhanced sialyltransferase activity, Table 23. An ATP regenerating system which consisted of ATP, phosphocreatine and creatine phosphokinase, was used in these incubations along with a protein kinase system, which consisted of cAMP and rabbit muscle cAMP dependent protein kinase. Magnesium was also included in incubations as a metal ion cofactor for both the ATP regenerating system as well as the protein kinase system. Removal of only ATP from the complete incubation mixtures resulted in effectively no enhancement of sialyltransferase activity, while removal

Figure 27. The effect of sodium fluoride (O) and sodium phosphate (●) on bovine intestine alkaline phosphatase activity.

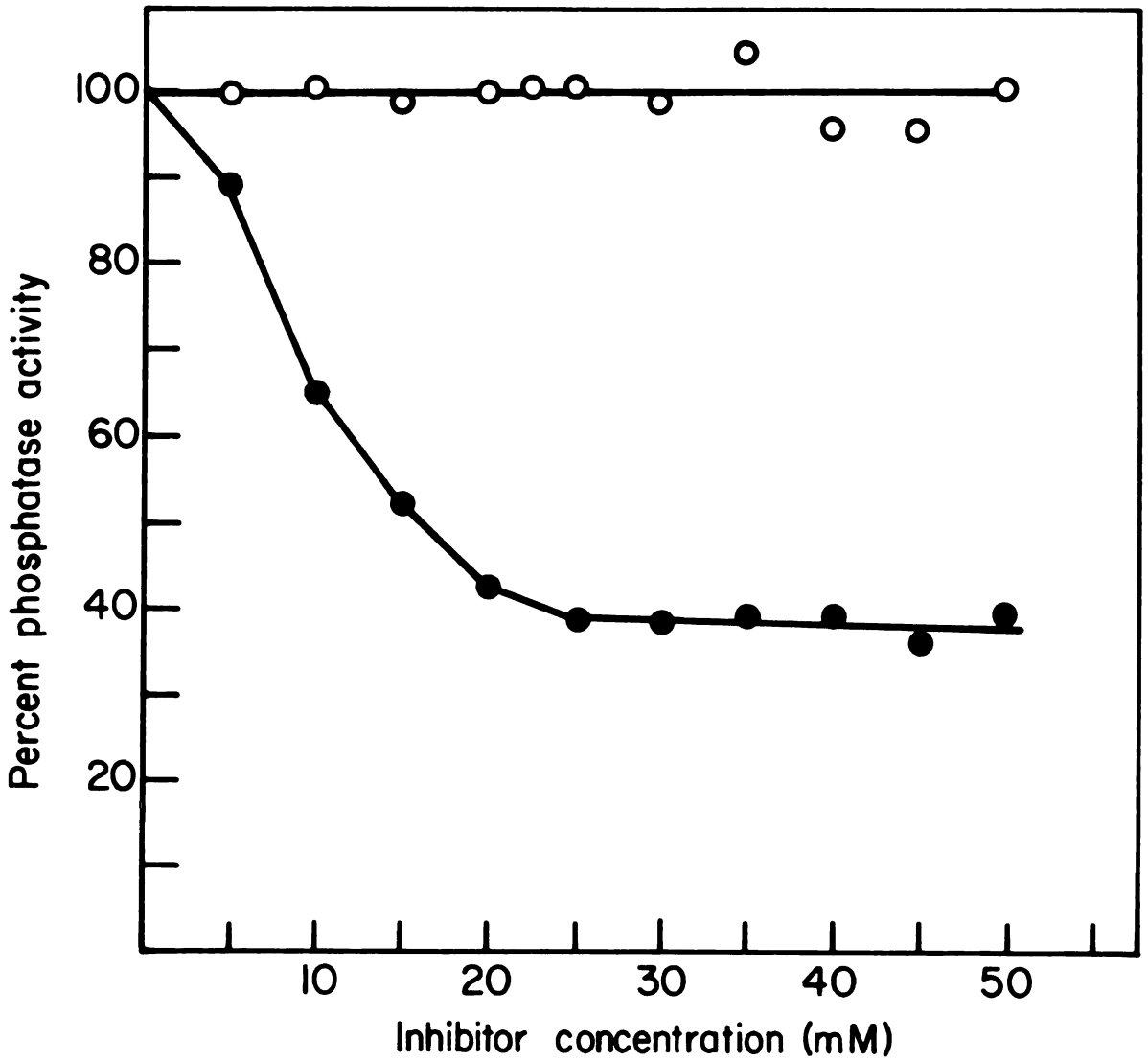

100
80
60
40
20
Percent phosphatase activity
10
20
30
40
50
Inhibitor concentration (mM)

Table 23. The effect of protein kinase on CMP-sialic acid:lactosylceramide sialyltransferase activity.

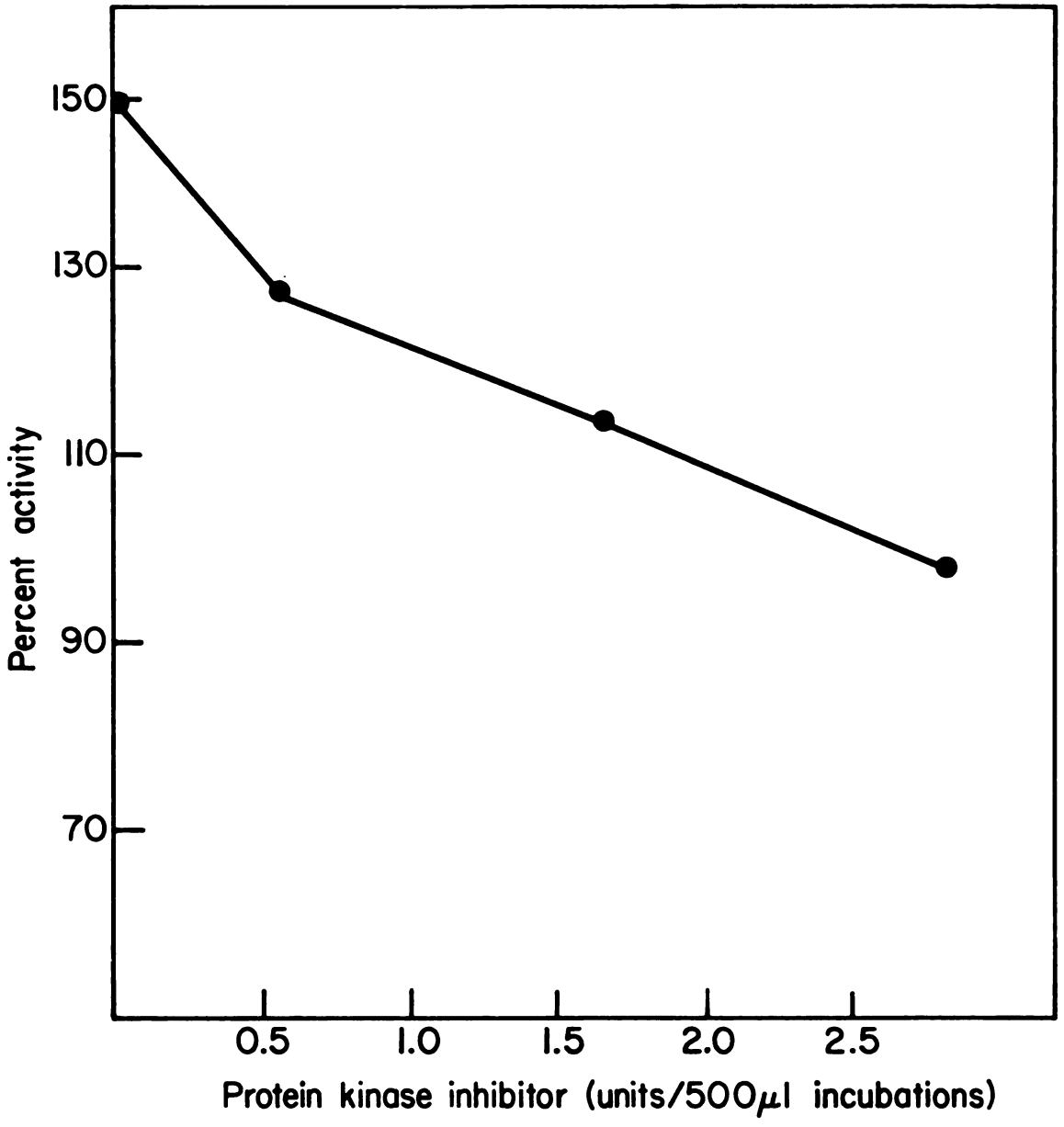
Preparation	Percent of Control
Control	100
Complete	155
minus ATP	107
minus ATP; plus hexokinase and glucose	87
minus ATP, phosphocreatine and creatine phosphokinase	89
minus cAMP dependent protein kinase	129
minus cAMP	133
minus cAMP and cAMP dependent protein kinase plus protein	126
plus protein kinase inhibitor	75

Complete incubations included ATP, phosphocreatine, creatine phosphokinase, cAMP and cAMP dependent protein kinase.

of the active ATP regenerating system resulted in slightly less activity than in controls treated in a similar fashion. Controls were without the ATP regenerating system and protein kinase system. When ATP was removed from incubations and hexokinase with glucose was added, a sialyltransferase activity very similar to controls was noted. Glucose with hexokinase has been used to remove ATP from other partially purified enzymatic systems [176].

Removal of cAMP, protein kinase or both from incubation mixtures resulted in levels of CMP-sialic acid:lactosylceramide sialyltransferase that were intermediate between complete (with the ATP regenerating system and the protein kinase system) and control (without both systems) incubations. This may indicate that the partially purified preparation contains an endogenous protein kinase. Dawson et al. [177] have demonstrated the presence of protein kinase activity in combined microsomal and Golgi membranes. Addition of rabbit muscle protein kinase inhibitor to complete incubations resulted in levels of sialyltransferase activity slightly below that of controls. Incubations of the complete mixture with increasing amounts of protein kinase inhibitor resulted in suppression of sialyltransferase activity when compared to that of complete mixtures, Figure 28.

Figure 28. The activity of CMP-sialic acid:lactosylceramide sialyltransferase incubated with protein kinase and various amounts of protein kinase inhibitor.


150
130
110
90
70
Percent activity
0.5
1.0
1.5
2.0
2.5
Protein kinase inhibitor (units/500μl incubations)

DISCUSSION

A general observation which can be made is that PMA stimulates cell division in most, but not all cell types under conditions where the cells have or can become growth-inhibited [127]. Under the conditions in V79 cell growth studies where PMA, but not non-promoting phorbol esters, stimulated growth at confluent densities, Yotti et al. [155] have shown that PMA inhibited intercellular communication. Since it is known that contact inhibition [177] seems to be mediated in part via intercellular communication [178-182], these results confirm the observation that growth occurs under conditions in which contact-mediated intercellular communication is inhibited. V79 cells were initially used since the effects of different phorbol esters on several properties of this cell line have been previously investigated [155].

Examination of GSL metabolism in PMA treated V79 cells indicates that tumor-promoting phorbol esters in the culture medium led to an enhanced level of gangliosides (mostly G_{M3}). This increase may be due to an increased ganglioside biosynthesis, decreased degradation or both. In addition phorbol ester treatment did not result in any structural changes of GSL. These data are strikingly similar to those observed upon thrombin treatment of human platelets [183]. Platelets display a simple ganglioside composition, the majority of which is G_{M3} [184]. Treatment of platelets with thrombin resulted in a two-fold increase in the concentration of ganglioside G_{M3}. This

change in GSL composition occurred in a very short time, 10 min, after the initiation of thrombin treatment. Thrombin treatment of platelets has long been known to activate protein kinase C [185], and recently it has been demonstrated that phorbol ester tumor-promoters bind to and activate protein kinase C [185]. Perhaps activation of protein kinase C is central to the mechanism by which ganglioside G_{M3} content is enhanced by thrombin and PMA treatment. Taken together the PMA and thrombin data indicate that CMP-sialic acid:lactosylceramide sialyltransferase may be a regulated enzyme in the GSL biosynthetic pathways. This enzyme, which catalyzes the formation of ganglioside G_{M3} (II^3-α-N-acetylneuraminyl-lactosylceramide), is of special interest and potential importance in regulation because it catalyzes the first committed step in the biosynthesis of nearly all gangliosides.

CMP-sialic acid:lactosylceramide sialyltransferase was characterized in NIL-8 cells since our laboratory previously characterized two other glycosyltransferases in this cell line, thus allowing for comparison of their properties [171,186]. Studies to characterize an assay for CMP-sialic acid:lactosylceramide sialyltransferase indicate that the enzyme's activity is relatively stable over a four hour period of time as compared to other glycosyltransferases [35,171,173]. In agreement with this finding, Kaufman and Basu [187] have demonstrated that a membrane preparation from homogenates of embryonic chicken brain contains enzymatic activities that catalyze the transfer of sialic acid to lactosylceramide (LacCer) and Gb4, and that the enzyme reaction with Gb4 can be reduced to ten percent of the initial activity by heating at 55°C for 30 sec, while very little change occurs in the activity toward lactosylceramide.

CMP-sialic acid:lactosylceramide sialyltransferase was found to require manganese for maximal activity *in vitro*. Other glycosyltransferases have been found to require manganese [35,171,187]. The explanation generally offered is that perhaps this requirement reflects localized concentrations of divalent metal cations. However, it is unlikely that such a localized high concentration of manganese exists within the Golgi apparatus, where carbohydrate addition to GSL has been demonstrated to occur [21,22,23]. Manganese may be a less specific substitute *in vitro* for another divalent cation. Manganese at high concentrations has been found to substitute for magnesium as a metal ion activator of the restriction enzyme Eco R1, for the enzyme DNA polymerase, and for *in vitro* RNA translation systems. The restriction enzyme preparation with manganese is referred to as Eco R1* and is less restrictive in cutting specific DNA sequences [188]. DNA polymerases with manganese are less specific for their choice of nucleotides to incorporate into DNA than they are with magnesium [189]. The translation systems with manganese are also less precise and as a result are error prone [190]. CMP-sialic acid:lactosylceramide sialyltransferase is a membrane-bound enzyme catalyzing the synthesis of a membrane component. Exogenous lactosylceramide added with detergent to assay tubes probably does not accurately approximate *in vitro* the conditions of lactosylceramide in Golgi membranes. Manganese, substituting for another metal ion activator, may allow the sialyltransferase to be less specific for the arrangement of exogenous lactosylceramide (substrate) in artifical membrane environments.

Several detergents, including Triton CF-54, had biphasic effects on CMP-sialic acid:lactosylceramide sialyltransferase activity;

increased enzyme activity was observed at low detergent concentrations while reduced activity was seen as higher concentrations. Low detergent concentrations affect most membrane-bound enzymatic activities [170], with optimal enzyme activity usually occurring when an enzyme is still membrane bound [170]. In many cases detergent effects may be due to the particulate nature of the enzyme preparations [191]. Detergents may merely alter conditions of the assay by making membrane vesicles permeable to substrates, products and effectors, and by changing the aggregation state of the membranes [191]. In some cases the detergent may also affect the state of the substrate [170]. It is difficult to determine whether the changes in activity result from the direct action of detergent on the enzyme molecule, on the membrane environment around the enzyme, on the substrate, or on all three. Both anionic and cationic detergents inactivated the enzyme. In contrast to the results with sialyltransferase, NIL-8 cell glycosphingolipid β-N-acetylgalactosaminyltransferase has a maximal enzyme activity with sodium taurodeoxycholate (0.1 percent w/v); other ionic detergents also gave increased activity [171]. It has been suggested that the different effects of membrane-perturbing reagents on Golgi membrane glycosyltransferase activities may be due to a different lipid environment around each transferase [192].

CMP-sialic acid:lactosylceramide sialyltransferase activity was highest at pH 6.5; and the use of different buffers at the same pH did not result in enzyme activity differences. This pH optimum for the sialyltransferase activity indicates that the lumen of the Golgi apparatus may have an acidic pH. Studies involving subcellular fractionation and isolation of Golgi membranes indicate that the Golgi apparatus

contains a proton pump capable of establishing a lumenal acidic pH [193]. However, the Golgi membrane preparations used in these studies contained lysosomal marker enzymes. The lysosome lumen has an acidic pH that is maintained by a well characterized proton pump [194].

Ganglioside G_{M3}, the enzyme reaction product, inhibited CMP-sialic acid:lactosylceramide sialyltransferse activity while other glycoconjugates were noninhibitory or inhibited activity to a lesser extent. The inhibition by G_{M3} ganglioside may reflect product inhibition or its use as an alternative (competitive) substrate for this enzyme. In the latter case, G_{D3} ganglioside would be expected to be synthesized by NIL-8 cells; however, no G_{D3} has been demonstrated to be present in NIL-8 cells [195]. Product inhibition of glycosyltransferases has been demonstrated in several systems [171]. In addition G_{M3} has been demonstrated to inhibit several glycosyltransferases [171]. It is interesting to note that Gb4 slightly inhibits CMP-sialic acid:lactosylceramide sialyltransferase and thus ganglioside G_{M3} formation; while ganglioside G_{M3} inhibits the glycosyltransferase which forms Gb4. Perhaps inhibition of other glycosyltransferases by ganglioside G_{M3} is a regulatory feature of GSL biosynthesis. The fact that desialized fetuin only slightly inhibits CMP-sialic acid:lactosylceramide sialyltransferase activity at high concentrations, indicates that desialized fetuin may not be an alternate substrate for sialic acid addition by this enzyme.

The requirement for detergent solubilization makes many conventional purification methods, such as ammonium sulfate percipitation and some kinds of chromatography unusable or of little value in protein purification. Detergent solubilization of proteins into micelles can

prevent proteins from interacting with ionic exchange groups and affinity ligands [175]. Membrane proteins solubilized at detergent concentrations above the critical micellar concentration (the CMC for Triton X-100 is approximately 0.016 percent w/v [196]) generally elute from size exclusion columns within a micelle. The approximate molecular weight of a Triton X-100 micelle is 90,000 [170]. Molecules not incorporated into micelles with molecular weights significantly different from protein/detergent micelles may be separated from such micelles by size exclusion chromatography. Since the membrane-bound sialyltransferase was solubilized from partially purified and washed (during the membrane concentration step) Golgi membranes, it is not surprising that little purification resulted after the solubilization step. Most proteins in the solubilization supernate were probably membrane-bound originally and then incorporated into micelles. Thus, subsequent purification steps resulted in relatively little further purification.

Phosphatidylcholine addition to the partially purified CMP-sialic acid:lactosylceramide sialyltransferase gave the greatest activation of the transferase, while moderate activation was also observed with phosphatidylethanolamine. Phosphatidylcholine is the most abundant phospholipid in Golgi membranes, while phosphatidylethanolamine is the next most abundant phospholipid [197]. Moreover, phosphatidylcholine and phosphatidylethanolamine are both positively charged at pH 6.5 and have similar structures. Phosphatidylserine, which inactivated the sialyltransferase, is also similar in structure to phosphatidylcholine and phosphatidylethanolamine, but has a carboxyl group attached to the common ethanolamine structure. Phospholipid activation of sialyltransferase may be due to partial replacement of enzyme boundary lipids.

Lysophospholipids were found to inactivate the CMP-sialic acid:lactosylceramide sialyltransferase. Lysophospholipids are known to behave as detergents [170,198]. With a single fatty acid at C-1 of the glycerol moiety they structurally resemble a detergent molecule with a charged (anionic) head group and a long hydrophobic tail [170]. Moreover, lysophospholipids in solution form micelles in equilibrium with monomers (as do detergents), while diacylphospholipids form liposomes [170,198]. Inactivation of the CMP-sialic acid:lactosylceramide sialyltransferase by detergent-like phospholipids is consistent with inactivation of sialyltransferase by ionic detergents (as demonstrated in this thesis).

The fact that phosphatidylcholine and phosphatidylethanolamine have equal but opposite effects when compared to lysophosphatidylcholine and lysophosphatidylethanolamine, indicates that choline and ethanolamine may be functional groups that are important for the interaction of phospholipids with the sialyltransferase. Phosphatidylcholine possibly is the primary activator of sialyltransferase and phosphatidylethanolamine a conservative replacement for phosphatidylcholine, due to the fact that addition of phosphatidylcholine to sialyltransferase assays resulted in much greater enzyme activity. In contrast to these results with sialyltransferase, other glycosyltransferases have been demonstrated to have increased activity in the presence of other phospholipids [20,35,171]. Galactosyltransferase from bovine milk, which transfers galactose from UDP-Gal to glucose and GalNAc has been shown to be stimulated by phospholipids [199]. Phosphatidylcholine activated the enzyme better than several other phospholipids. Phosphatidic acid and phosphatidylserine inhibited the activity almost

totally, while phosphatidylinositol had no effect. Although milk galactosyltransferase is not considered a membrane-bound enzyme, it exists in a lipid-rich environment in milk which may be similar to the environment of a membrane-bound Golgi apparatus transferase. This is consistent with the hypothesis that the various types of glycosyltransferases may have a characteristic membrane lipid environment, and that the varied effects of detergents on Golgi membrane glycosyltransferase activities may be due to the characteristic lipid environment around each type of transferase [192].

Phosphatidylcholine as a boundary lipid may in part regulate sialyltransferase activity. For example, 3'-sulfogalactosylceramide is believed to complex with Na^+K^+ ATPase and serve as a K^+ selective factor. Indeed, Paddon and Vance [200] have demonstrated that PMA treatment of cultured cells increases phosphatidylcholine biosynthesis. Such an increase could modulate CMP-sialic acid:lactosylceramide sialyltransferase activity. Alternatively, the phospholipids may affect the state of the substrate, lactosylceramide. Exogenous lactosylceramide added with detergent probably does not accurately approximate *in vitro* the conditions of lactosylceramide in Golgi membranes. Phosphatidylcholine and phosphatidylethanolamine may allow lactosylceramide to arrange itself in a suitable configuration for the sialyltransferase to act upon it. Studies on glycolipid synthesis in bacteria suggest a mode of interaction for phospholipids with substrate and enzymes. Rothfield and Pearlman [201] have found that two purified microbial glycosyltransferases have an absolute requirement for phospholipids. Further studies have revealed that a glycolipid-phospholipid complex is formed which then interacts with the enzyme [202], and

it has been suggested that this complex provides a hydrophobic environment which favors the transfer of sugars to glycolipids [203]. It is difficult to determine whether the changes in transferase activity result from the direct action of phospholipid on the enzyme molecule, on the substrate or both.

Regulation of CMP-sialic acid:lactosylceramide sialyltransferase activity during cell cycle was observed. The sialyltransferase was found to exhibit activity throughout the cell cycle; however, peak activities were found in M/early G_1 and G_1 phases. During M/G_1 phase the cells were distributed over M and G_1 phases of cell cycle. From the data it is not possible to determine if the increased sialyltransferase activity occurs while the cells are in mitosis or early G_1 phase. Cells in G_2 and S phases were found to have lower sialyltransferase activity. These results confirm other work demonstrating that glycosphingolipid metabolism is affected by cell cycle and cell density [62]. Ceramide tri, tetra-, and penta-saccharides of NIL cells were maximally labeled during the G_1 phase and minimally during the S phase, when the cell surface labeling techniques of galactose oxidase-sodium borotritiide were utilized; the actual amounts of these lipids remained relatively constant [204]. Experiments with human epithelial KB cells demonstrated that glycolipid and glycoprotein synthesis were cell cycle specific. Maximum incorporation of [^{14}C]-galactose into glycosphingolipids occurred in M and early G_1 phases and into glycoproteins in late S and early G_2 phases [205]. Sodium butyrate, which blocks the cell-cycle in early G_1 phase [206], was shown to induce CMP-sialic acid:lactosylceramide

sialyltransferase activity [207,208], also indicating that glycosphingolipid metabolism may be regulated during cell growth cycle.

CMP-sialic acid:lactosylceramide sialyltransferase activity increased as NIL-8 cells reached density dependent growth inhibition. Cell contact-dependent enhancement of glycolipid synthesis has been demonstrated in several cultured cell types [62]. Increased glycosphingolipid synthesis was more easily demonstrated by [^{14}C]-galactose or [^{14}C]-palmitate incorporation than by analysis of chemical quantity. This suggests that cell-to-cell contact induces not only net synthesis but also metabolic turnover of glycosphingolipids [209]. UDP-galactose:lactosylceramide α-galactosyltransferase activity is increased several fold in confluent BHK or NIL cells when compared to sparsely growing cells. However, not all enzyme activities involved in the biosynthesis of glycosphingolipids are increased upon cell-cell contact. For example, the synthesis of lactosylceramide, catalyzed by UDP-galactose:glucosylceramide β-galactosyltransferase, is not enhanced when cells reach confluence [13].

Treatment of the partially purified CMP-sialic acid:lactosylceramide sialyltransferase with bovine intestinal alkaline phosphatase resulted in a reduction of the sialyltransferase activity, while treatment with protein kinase resulted in an enhanced sialyltransferase activity. It appears from these data that CMP-sialic acid:lactosylceramide sialyltransferse activity may also be regulated by covalent modification of the enzyme. If so, the results presented in this thesis indicate that phosphorylation enhances enzymatic activity, while dephosphorylation suppresses enzymatic activity. When the partially purified sialyltransferase was incubated with increasing amounts of

bovine intestinal alkaline phosphatase, sialyltransferase activity decreased and eventually reached a plateau. This plateau in activity was approximately a thirty percent decrease in activity. While some enzymes have been demonstrated to be totally inhibited by dephosphorylation, several other enzymes believed to be regulated by phosphorylation-dephosphorylation have not been demonstrated to be totally inactivated by dephosphorylation. Phosphatase treatment of phenylalanine hydroxylase has been found to only decrease hydroxylase activity to forty percent of controls [210]. The final hydroxylase activity reached a plateau after an extended period of phosphatase treatment. Dephosphorylation of RNA polymerase has been demonstrated to reduce polymerase activity by approximately fifty percent [211]. Moreover, hydroxylmethylglutaryl CoA (HMG-CoA) reductase is believed to be inactivated by phosphorylation and activated by dephosphorylation. Several studies using rat liver microsomal reductase revealed that inactivation under phosphorylating conditions was prompt but never complete [212,213]. It has been postulated that this reduced activity represented "a maximal state of inactivation" of HMG-CoA reductase. Enhancement of sialyltransferase activity by protein kinase treatment was only fifty percent higher than control levels. Similarly, _in vitro_ phosphorylation of rat liver fructose-1,6-bisphosphatase by the catalytic subunit of cAMP dependent protein kinase from bovine intestine resulted in only a forty percent increase in fructose-1,6-bisphosphatase activity [214]. There may be several reasons for this: (a) an exogenous protein kinase is being used which is not the normal phosphorylating agent, and complete phosphorylation of all the enzyme molecules did not occur; (b) the sialyltransferase has been

solubilized from its membrane and the membrane may be important for regulating enzyme conformation to allow optimal enzyme phosphorylation; and (c) perhaps phosphorylation increases enzyme activity and reduces enzyme degradation, resulting in a higher net enzyme activity *in vivo*.

Solubilization and partial purification of CMP-sialic acid:lactosylceramide sialyltransferase eliminates several alternative explanations for enhanced activity by protein kinase treatment and suppressed activity by phosphatase treatment. Transport of CMP-sialic acid into Golgi vesicles has been demonstrated *in vitro* to be a carrier mediated process [36]; and the possibility was considered that this is the rate-limiting step for the production of ganglioside G_{M3} from lactosylceramide *in vivo* by the Golgi apparatus. Solubilization of CMP-sialic acid:lactosylceramide sialyltransferase from Golgi membranes excludes sugar nucleotide transport as an alternative mechanism for modulation by kinase and phosphatase of sialyltransferase activity in these experiments. Phospholipids have been demonstrated to alter sialyltransferase activity in this thesis as well as other glycosyltransferase activities by other investigators [20,35,171]. Removal of membrane lipids from the enzyme eliminates alteration of enzyme boundary lipids as a modulator of sialyltransferase activity. Arce *et al*. [215] have previously demonstrated that glycosyltransferases in subcellular particles of rat brain were not able to freely interact with all of the GSL substrates in the preparation. Thus, substrate availability may play a significant role in determining the rate of GSL synthesis *in vivo*. Solubilization of the sialyltransferase also eliminates this factor *in vitro*.

Recognition that reversible covalent modification of proteins is a major regulatory process followed from the studies of glycogen metabolism. Glycogen phosphorylase was shown to exist in two interconvertable forms, now known to be the nonphosphorylated and phosphorylated forms of the enzyme. Since elucidation of this first example, other reversible covalent protein modifications have been described including acetylation-deactylation, adenylylation-deadenylylation, uridylylation-deuridylylation and methylation-demethylation [216]. In addition, the scope of this particular type of regulatory mechanism has broadened to include nonenzymatic proteins.

For phosphorylation-dephosphorylation of enzymes to serve a regulatory function, these conversions must themselves be regulated. This requires control in the activity of protein kinases and/or phosphatases. The kinases are often regulated by specific effector compounds or messengers. The phosphoprotein phosphatases are regulated, not through direct interaction with specific effectors, but instead by substrate directed effects, in which a metabolite or metal ion combines with the phosphorylated enzyme substrate causing it to become a better or poorer substrate for the phosphatase. Phosphoprotein phosphatases are also regulated by interactions with modifier proteins such as phosphatase inhibitors. In vivo and in vitro studies indicate that interconversion of an enzyme between active and inactive forms is a dynamic process that leads to steady state, and that the distribution of modified and unmodified forms of the enzyme is governed by the activities of the kinase and phosphatase.

CMP-sialic acid:lactosylceramide sialyltransferase is a good candidate to be a regulated enzyme in the GSL biosynthetic pathways for

several reasons: (a) its activity is regulated during the cell cycle as is GSL biosynthesis [62]; (b) its *in vitro* activity appears to be rate limiting when compared to other glycosyltransferases [20,35,171]; (c) its GSL substrate (lactosylceramide) and GSL product (ganglioside G_{M3}) are key intermediates that initiate several alternative GSL biosynthetic pathways [8,15,20]; (d) its product (G_{M3}) is a specific inhibitor of other GSL glycosyltransferases [171]; and (e) it catalyzes the first committed step of ganglioside biosynthesis [8,15].

Additionally, alterations of CMP-sialic acid:lactosylceramide sialyltransferase activity or in the levels of LacCer and G_{M3} have been demonstrated in several cultured cell lines where alterations of protein phosphorylation also occur. PMA is thought to bind to and activate protein kinase C, which is capable of phosphorylating other proteins [185]. Studies described in this thesis demonstrate that PMA treatment of cultured cells results in changes in both GSL and sialyltransferase activity. Thrombin treatment of human platelets is known to result in a reduction of lactosylceramide and a corresponding increase in ganglioside G_{M3} with a half time of five minutes. Moreover, thrombin treatment of platelets is known to activate protein kinase C. Insulin treatment of 3T3-C2 cells results in an increased cellular content of ganglioside G_{M3} and G_{D1a} [115]. This clone of 3T3 cells does not differentiate into adipocytes, thus the alteration in ganglioside content is due to insulin treatment and not differentiation [102]. Insulin treatment of cultured cells has been demonstrated to result in increased phosphorylation of several proteins [217]. Purified insulin receptor has been found to phosphorylate both

itself [218] and other proteins [217] upon the addition of insulin. The insulin-stimulated protein kinase activity co-purifies with the insulin receptor during (a) immunoprecipitation with anti-insulin receptor antibodies [219] and (b) affinity chromatography on insulin-Sepharose [220]. Furthermore, the insulin receptor can be labeled using ATP affinity reagents [221,222]. Together, these insulin receptor studies indicate that the receptor is a protein kinase, and that insulin activation of this protein kinase results in increased ganglioside G_{M3} biosynthesis. In other cells, cAMP has also been demonstrated to increase ganglioside G_{M3} content [223]. Cyclic nucleotides are well known activators of cyclic nucleotide dependent protein kinases, which may then phosphorylate other proteins [224]. Thus, there is correlative evidence which suggests that CMP-sialic acid:lactosylceramide sialyltransferase activity may be regulated by phosphorylation-dephosphorylation.

The studies described in this thesis have been designed to address the broad question of how GSL biosynthesis is regulated. The results, when examined with a variety of other studies, may help to understand the mechanisms involved in the regulation of GSL biosynthesis, the regulation of plasma membrane biosynthesis, as well as the function of GSL in the plasma membrane.

ADDENDUM

The author would like to report that since the writing of this dissertation attempts to activate the sialyltransferase using protein kinase have failed. ATP alone seems to be sufficient to activate the partially purified enzyme. Potential problems with reagents were eliminated since fresh ones were tried. Procedures to isolate Golgi membranes and to detergent-solubilize them have been repeated. The problem may occur during cellulose-phosphate chromatography of the solubilized sialyltransferase. Formerly, as reported in this dissertation, the solubilized enzyme did not interact with the cellulose-phosphate and quantitatively passed through the column. In recent studies, quantitative recovery of the enzyme has not been achieved. The eluted enzyme activity appears to be less stable.

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June 19, 1984

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