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GLYCOLIPID GLYCOSYL TRANSFERASES OF A HAMSTER CELL LINE IN CULTURE

I. KINETIC CONSTANTS, SUBSTRATE AND DONOR NUCLEOTIDE SUGAR SPECIFICITIES

K.A. CHANDRABOSE and I.A. MACPHERSON

Department of Tumour Virology, Imperial Cancer Research Fund Laboratories, P.O. Box No. 123 Lincoln's Inn Fields, London, WC2A 3PX (U.K.)

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Summary

The properties of enzymes catalysing the transfer of a galactose from UDP-galactose to exogenous ceramide monohexoside and ceramide di-hexoside derived from the Syrian hamster cell line NIL 2 were studied. The products of these enzymes were characterized by chemical and enzymatic methods.

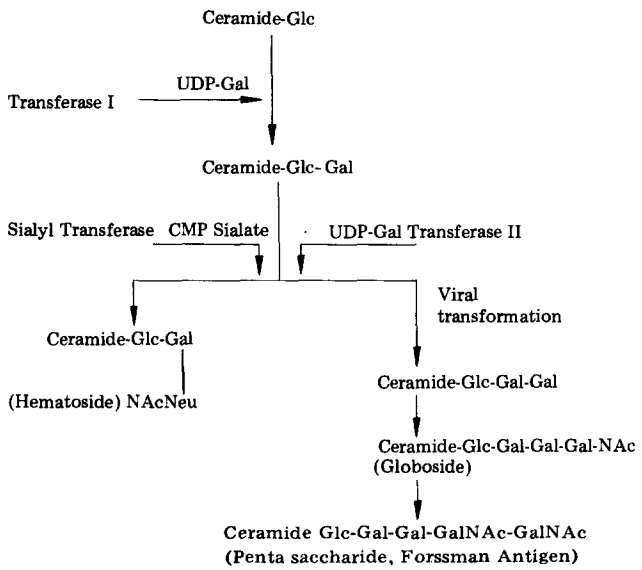
Kinetic analyses showed that the enzymes are susceptible to inhibition and activation by a number of substrate analogues. The kinetic and inhibition constants, glycolipid substrate specificity and nucleotide sugar donor specificity have been studied.

Introduction

Malignant transformation results in a variety of alterations in animal cell surfaces [1—3] and it is generally believed that one or more of these changes may have a key role in the control of cell multiplication. Previous studies from this [3—5] and other laboratories [6,7] have shown that the synthesis of certain glycosphingolipids which are present in the plasma membrane [4], is reduced or blocked following viral transformation probably because the addition of sugars to the precursor molecules of these glycolipids does not take place.

The glycosphingolipids found in the NIL2 hamster cell line and a possible bio-synthetic pathway [8,9] are shown in Scheme 1. The tri-, tetra-, and penta-glycosyl ceramides which disappear as a result of viral transformation were shown to increase in the untransformed cells with increasing culture density [3,5].

SCHEME I



The scheme of synthesis as suggested by Roseman [9]. The structure of glycosphingolipids as reported by Hakomori et al. [8].
Abbreviations: Reactions catalysed by transferase I and transferase II are referred to in text as reactions I and II respectively.

The transferase enzymes responsible for the synthesis of these saccharide chains are generally believed to be highly selective and to require specific nucleotidyl sugar donors [9].

There have been several studies on these enzymes from different sources. A glucosyl and a galactosyl transferase from embryonic chicken brain have been characterized by Basu et al. [10] and Hildebrand and Hauser [11] have reported on the properties of these transferases in extracts of rat spleen and kidney. More recently Yeung et al. [12] reported on the in vitro synthesis of neutral glycolipids from adrenal tumor cells. The results of studies by Kijimoto and Hakomori [13] on the transferase from NIL2 cells agree with those obtained in the present study which was undertaken as part of a more extensive appraisal of the role of cell surface glycolipid metabolism on animal cell growth regulation.

Experimental Procedure

Materials

Lactosyl ceramide was from Miles Laboratories Ltd., England. Other glycosphingolipids were purified from pig lungs and horse spleen. Radioactive UDP-galactose and UDPglucose labelled with ^{14}C in the carbon 1 position were from The Radiochemical Centre, Amersham, England. Radioactive UDP-*N*-acetyl-[1-^{14}C]galactosamine labelled on carbon-1- of the galactosamine moiety, was

from New England Nuclear. Nucleotide sugars were stored at −20°C and their stability checked by paper chromatography every 2 months. Unlabelled nucleotide sugars used as carriers, the phospholipids, ribonucleotide triphosphates and cyclic AMP, were from the Sigma Chemical Co. Triton X 100 was from British Drug House, England, and "Cutscum" a gift from Dr. S. Hakomori.

Cell culture

Conventional methods of cell culture were used, with clones of the NIL 2-Syrian hamster line, and a derivative clone NIL-2-HSV, transformed by Hamster Sarcoma Virus [5].

Enzyme assays

(a) Preparation of particles. Confluent cultures grown in 2.5L roller bottles were used throughout, except for experiments specifically designed to test the effect of cell density on enzyme activity. After removing the medium the monolayer of cells was washed once with isotonic phosphate buffer, pH 7.0. Cells were scraped into buffer and centrifuged. Cell pellets were either used immediately or stored at −20°C. Frozen cells retain their enzyme activities for 3 months.

When fresh cells were used they were suspended in 4 vol. of 0.32 M sucrose containing 0.014 M 2-mercaptoethanol and 0.01 M EDTA pH 7.0 and either homogenized in a hand homogenizer or sonicated with an immersible probe at an output of 80w for 15 s, (Sonioprobe, Type 1130A, Dawe Instruments Ltd.). Suspensions were examined microscopically for complete cell breakage, before using the homogenate for further processing. Nuclear breakage as a result of sonication has no effect on enzyme activities. Frozen cells were thawed at room temperature and processed as fresh cells. Homogenates were used directly for some experiments, whereas in others a crude microsomal pellet prepared as described by Kijimoto and Hakomori [13] was used. All operations during the preparation of particles were conducted at a temperature not exceeding 4°C.

(b) Incubation mixture. The substrate glycolipid (ceramide monohexoside or ceramide dihexoside) was prepared for incubation as follows. An aliquot of a chloroform/methanol (2 : 1) solution of the glycolipid of known concentration was evaporated to dryness. A solution of IM cacodylate-HCl buffer at pH 6.1 containing either Triton X-100 or 'MCutscum" at a concentration of 13.6 mg/ml (w/v) was added to the dry glycolipid and sonicated in an ultrasonic bath. The solution was heated to 45°C for 10 min and cooled to room temperature. Aliquots of this solution were pipetted to give glycolipid concentrations ranging from $5 \cdot 10^{-4}$ to $2.5 \cdot 10^{-2}$ μmol in individual assay tubes. The total volume of glycolipid-cacodylate-detergent mixture was kept constant at 60 μl/200 μl of incubation mixture, so that the detergent concentration did not exceed 850 μg in the mixture. To this mixture were added $MnCl_2$ and UDP-galactose to final concentrations of 150 and 25 mM, respectively; 100 μl of the enzyme preparation (0.4—0.8 mg protein) to make a total volume of 200 μl.

The reaction was stopped by the addition of 4 ml of chloroform/methanol (2 : 1) and after standing at room temperature for 2 h, the reaction mixture was partitioned with 1/5 vol. of water and upper layer was discarded. The interphase was washed twice with 1 ml of 0.1 M KCl and the organic phase

evaporated to dryness and analysed on silica gel G thin layer plates (E. Merck and Co.) by ascending chromatography in chloroform/methanol/water (65 : 25 : 4) [5].

The thin layer plates were autoradiographed and the spots corresponding to known standards of ceramide dihexoside and ceramide trihexoside were located, scraped and measured by scintillation spectrometry.

Identification of reaction products

The radioactive spot corresponding to ceramide dihexoside was eluted and chromatographed. It produced a single radioactive spot with an R_F value corresponding to standard ceramide dihexoside [5]. No further characterization of this compound was attempted. The spot corresponding to ceramide trihexoside when subjected to the same procedure as above, gave three radioactive spots of which only one had the R_F value of standard ceramide trihexoside in the 2nd dimension when the two dimensional chromatographic system previously reported [5] was used. Further characterization of this compound was made as follows. The radioactive spot corresponding to ceramide trihexoside in one dimensional chromatography using chloroform/methanol/water (65 : 25 : 4) was scraped off and eluted. It was saponified with 1 M methanolic sodium hydroxide for 1 h at room temperature followed by neutralisation with aqueous HCl, and partitioning. The organic phase was chromatographed in three different solvent systems. Autoradiography of the plates gave one radioactive spot in each solvent, as shown in Fig. 1.

The radioactivity of the biosynthesized ceramide trihexoside was shown to be associated only with the terminal galactose as shown by its loss during hydrolysis of the material with fig α-galactosidase prepared according to the method of Hakomori et al. [14]. The radioactive material isolated from thin-layer chromatography plates shown in Fig. 1 was pooled and incubated with galactosidase [14]. The radioactivity released amounted to 70% when the material was co-chromatographed with known galactose (Fig. 2). The remaining

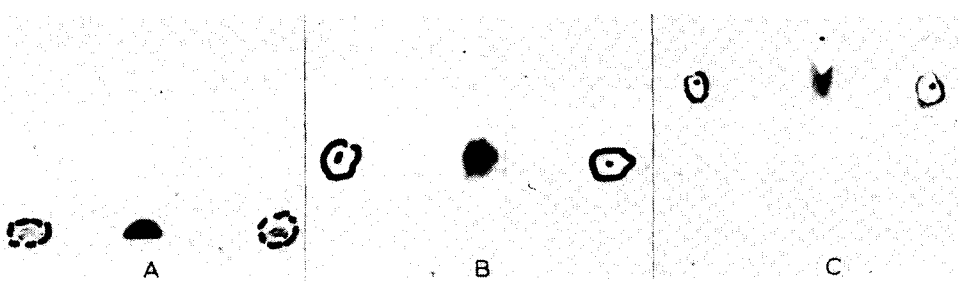


Fig. 1. Autoradiograph of thin layer chromatogram of biosynthesized ceramide trihexoside in three different solvent systems. The reaction mixture was processed as described in the text. After saponification and chromatography on silica Gel G plates (in solvent system 10 : 6 : 4 : 1, tetrahydrofuran/methylal/methanol/water). The plates were radioautographed and the only radioactive spot present corresponded with standard ceramide trihexoside. This spot was eluted, divided into three aliquots and chromatographed again in three different solvents and autoradiographed. The circle on either side of each radioactive spot represents the positions of standard ceramide trihexoside. Solvent systems used were: A, 1-propanol/water/conc. ammonium hydroxide (80 : 13.3 : 67); B, Chloroform/methanol/conc. ammonium hydroxide (60 : 40 : 10); C, Chloroform/methanol/water (65 : 25 : 4).

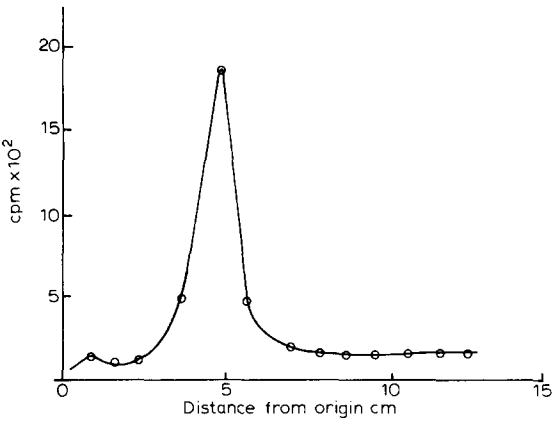


Fig. 2. Chromatography of galactose released from biosynthesized ceramide trihexoside by fig α-galactosidase. The biosynthesized ceramide trihexoside purified as given in Materials and Methods was incubated with α-galactosidase, according to the method of Hakomori et al. [14]. The sugar was extracted from upper phase with hot pyridine and applied to cellulose plates (MN300, E. Merck and Co.) in 10% isopropanol. Plates developed in ethyl acetate/pyridine/water (2 : 1 : 2). The peak of radioactivity was found to correspond with standard galactose.

30% was accounted for as increased ceramide trihexoside by chromatography of the lower phase.

The identity of the product was further confirmed by the production of a single precipitin line upon immunodiffusion analysis with rabbit antisera immuno-specific for ceramide trihexoside *. When chromatographed in the 65 : 25 : 4 system about 15—30% of the counts contaminating the ceramide trihexoside were found to be saponifiable material. No attempt was made to characterize these materials.

Because of the contamination with saponifiable material the lower phase was saponified and chromatographed in all routine assays in 65 : 25 : 4 (chloroform/methanol/water). Products located by autoradiography were also compared with known standards and then scraped and counted, as gelled samples using Carbosil. Counting efficiencies were determined using [^{14}C]toluene as internal standard. Protein was determined according to the method of Lowry et al. [15].

Preparation of labelled glycolipid substrates

Labelled ceramide dihexoside and ceramide trihexoside were prepared by growing NIL-2 cells in the presence of [^{14}C]palmitate and isolating the compounds by two dimensional chromatography [3,5].

Recovery of glycolipids from the assay mixture

Labelled glycolipids added to mock incubation systems yielded 88—92% of the original counts when chromatographed as described. All reported data is corrected assuming a general recovery of 90%.

* Kindly performed by J. Collins and A. Destree

Results

Requirements for enzyme activity

As shown in Table I optimal reaction rates were obtained only with the complete system given in the methods. Substituting manganese ions with magnesium resulted in a 50% reduction in activity in reaction I, while it reduced the rate of reaction II by 80%. "Cutscum" detergent gave the same results as Triton X-100 in both reactions. Both detergents when used above concentrations of 4 mg/ml strongly inhibited both activities.

Both reactions had an absolute specificity for UDP-galactose, as the nucleotide sugar donor. When UDP-[1-^{14}C]glucose was used the di- and tri-glycosyl ceramides were not labelled, but heavy endogenous labelling appeared in ceramide monohexoside.

There was reduction in labelling in ceramide dihexoside and ceramide trihexoside when unlabelled UDP-glucose was added at the same concentrations as UDP-[1-^{14}C]galactose in the complete system. With UDP-*N*-[1-^{14}C]acetylgalactosamine as the radioactive sugar donor, 10—14% of the counts found in complete incubation system were found in regions where ceramide dihexoside and ceramide trihexoside were chromatographed in 65 : 25 : 4. However with UDP-[1-^{14}C]galactose as the sugar donor, apart from the ceramide dihexoside and ceramide trihexoside formed as indicated in Table I, labelling was also found in two spots corresponding to R_F values of tetra and penta glycosyl ceramides. This unexpected observation is discussed later.

TABLE I

REQUIREMENTS FOR ENZYME ACTIVITY AND SUGAR DONOR SPECIFICITY

The complete system is given in the Experimental Procedure. Reaction was stopped after one hour and the mixture was processed as described in the text. The microsomal pellet prepared as described was used as the enzyme source.

Incubation mixture	Labelled glycolipid synthesized in pmol · mg protein^{-1} · h^{-1}	
	Ceramide dihexoside synthesized	Ceramide trihexoside synthesized
Complete	780	650
No detergent	130	98
No glycolipid substrate	168	159
Plus EDTA (5μmol)	15	9
No active + heat inactivated enzyme	27	18
No UDP [1-^{14}C]galactose + UDP-[1-^{14}C]glucose at the same concentration	165	86
No UDP-[1-^{14}C]galactose + UDP-[1-^{14}C] GalNAc (0.2 μCi. Specific activity 52 μCi per μmol)	110*	102*

* These numbers were calculated on the basis of dpm obtained from areas of thin layer plates where ceramide dihexoside and ceramide trihexoside chromatograph in the solvent system. No attempt was made to characterize them.

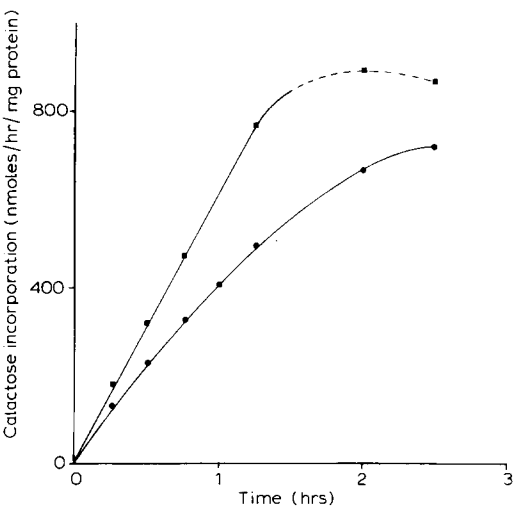


Fig. 3. Effect of incubation time on rate of formation of ceramide dihexoside and ceramide trihexoside. Conditions were the same as given in Table I. The reactions were stopped at indicated intervals of time and mixture processed as given in the text. ■———■, ceramide dihexoside synthesis; ●———●, ceramide trihexoside synthesis.

Effect of changes of protein concentrations, pH, time and temperature of incubation on rates of reactions

The effect of time on reaction is shown in Fig. 3, and that of changing protein concentration in Fig. 4. A pH optimum of 5.9 to 6.1 was observed for reaction I and 6—6.4 for reaction II. Both assays were therefore routinely run at pH 6.1. The temperature sensitivity of the enzymes differed markedly. Incubation at 45° C for 10 min completely abolished the formation of any detectable trihexoside, while the same treatment did not reduce the rate of dihexoside formation by more than 20%.

Kinetic constants of the reactions

The effect of varying the concentration of UDP-galactose on both reactions is shown in Fig. 5 as the Lineweaver-Burke plot of the same data. From this data the approximate K_m values for UDP-galactose are $2.2 \cdot 10^{-3}$ M for reaction I and $0.57 \cdot 10^{-3}$ M for reaction II.

The effect of glycolipid substrate concentration on reaction rates is shown in Fig. 6 and 7. Both reactions are strongly inhibited by their own substrates at concentrations higher than 100 μM. In a previous study on the same enzymes, no mention is made of this inhibition. The same transferases from rat spleen apparently do not show substrate inhibition even at much higher concentration of the glycolipid [11]. However, inhibition of reaction velocities by excess substrate on membrane bound enzyme systems of sphingolipid biosynthesis has been reported by others [16,17].

In all assays when the total enzyme levels of a culture were determined a variety of substrate concentrations were tried and only the maximal velocities obtained before inhibition were taken as the V_{max}. Even under such experimental conditions it is clear (See Fig. 6 and 7) that in the case of transferase II

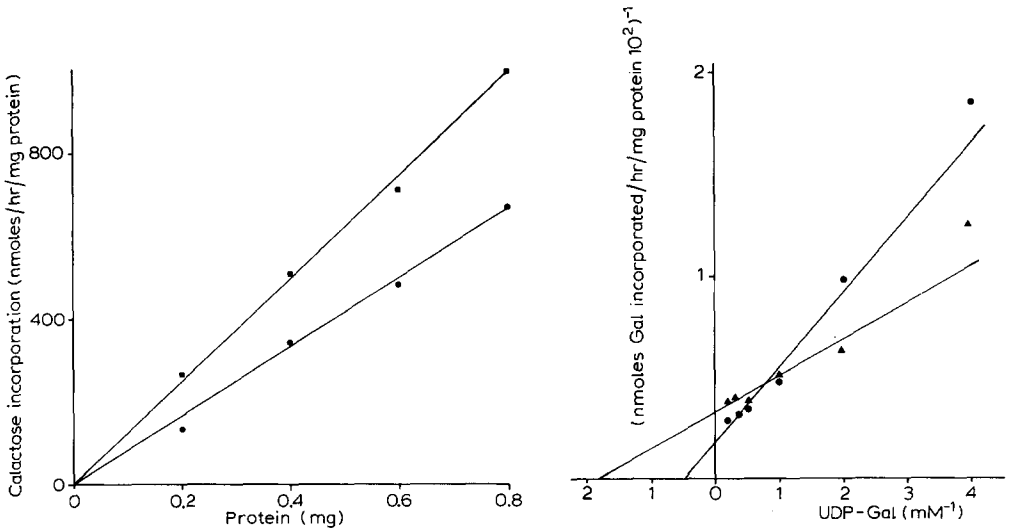


Fig. 4. Effect of protein concentration on the rate of formation of ceramide dihexoside and ceramide trihexoside. The rate is linear between 0.2 to 1.8 mg of protein. ●——●——●, Rate of formation of ceramide dihexoside; ■——■——■, rate of formation of ceramide trihexoside.

Fig. 5. Lineweaver-Burk plots for reactions I and II. Reaction conditions were as given in Table I but UDP-gal concentrations were varied. ●——●——●, Conversion of ceramide monohexoside to ceramide dihexoside. Calculated K_m value for UDP-gal $2.2 \cdot 10^{-3}$ M. ▲——▲——▲, Conversion of ceramide dihexoside to ceramide trihexoside and inhibition by ATP, K_m for UDP-Gal = $0.57 \cdot 10^{-3}$ M.

which is strongly inhibited by its substrate, the V measured experimentally falls well short of the true velocities attainable. The inset of Fig. 7 shows the normalised curve for the same data when $\sigma = S/K_m$ and $\phi = v/V$ and where S is the substrate concentration and v the corresponding observed velocity. The extent to which ϕ fails to reach unity is a measure of the inhibitory effect exerted by

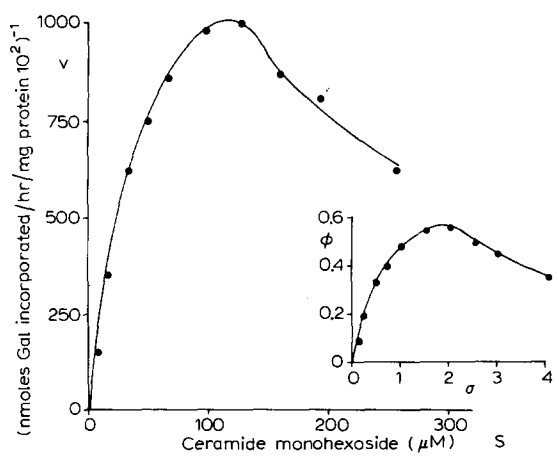


Fig. 6. Effect of concentration of ceramide monohexoside on formation of ceramide dihexoside. Incubation conditions were the same as given in Table I, except that the concentration of ceramide monohexoside was varied as indicated. The inset shows the normalised curve for the same data where $\sigma = [S]/K_m$ and $\phi = v/V$.

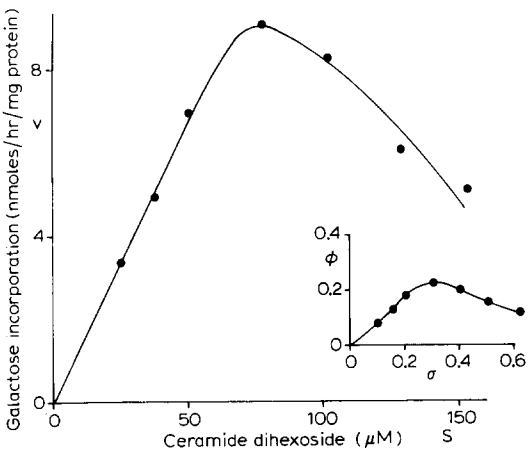


Fig. 7. Effect of ceramide dihexoside concentration on the formation of ceramide trihexoside. Conditions of incubation were the same as given in Table I, except that concentrations of ceramide dihexoside were varied as indicated. The inset is the normalised curve for the same data (See legend for Fig. 6).

the substrate. The value of V_{max} was taken from the reciprocal plots shown in Figs. 8b and 9b because this plot spreads out the data for lower substrate concentrations so that the greater part of the curve represents the region where the Michaelis law is obeyed [18]. Figs. 8a and 9a show the apparent inhibition constants obtained for both enzymes, as well as their K_m values for their glycolipid substrates. Because of the limited solubility of the glycolipid substrates in aqueous media the enzyme-substrate interaction is probably taking place with the substrates in their micellar condition in an in vitro assay system.

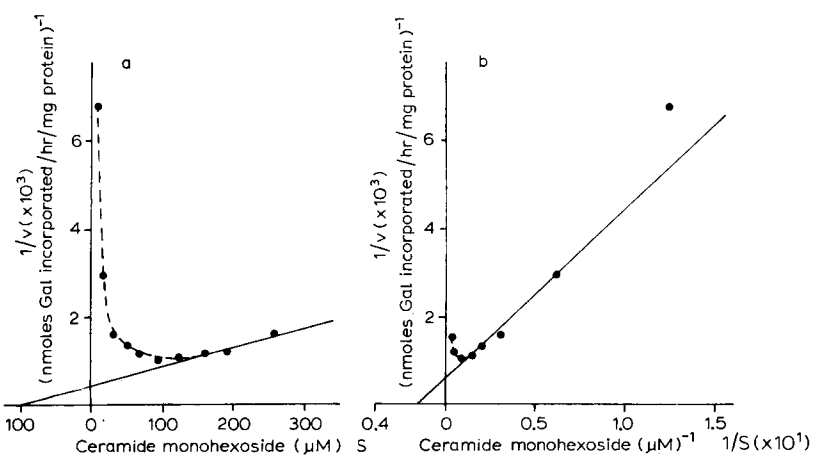


Fig. 8. (a) Determination of inhibition constant from inhibition by excess substrate for reaction I. (b) Lineweaver-Burk plot for the same data. Conditions of experiment were the same as given in Table I, except that concentration of ceramide monohexoside was varied. Calculated K_m value for ceramide monohexoside = $6.25 \cdot 10^{-5}$ M. Inhibition constant for ceramide monohexoside = $1.08 \cdot 10^{-4}$ M. (Equations IV.43—IV.45 in ref. 18, page 75, forms the basis for the treatment of kinetic data for evaluation of inhibition constant shown in (a). The data holds only if it is assumed that at higher concentrations of substrate, the substrate-detergent micelle formed is no longer the active species that can bind the enzyme effectively as in low substrate concentrations).

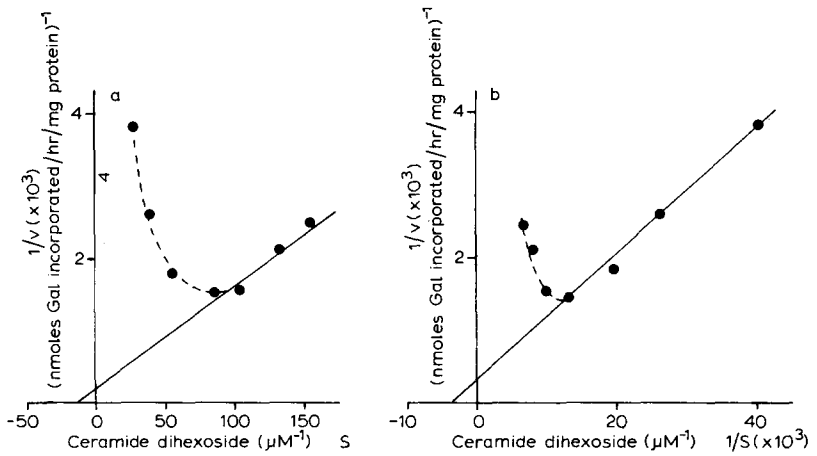


Fig. 9. (a) Determination of inhibition constant from inhibition by excess substrate for reaction II. (b) Lineweaver-Burk plot for the same data. Conditions of experiment were the same as given in Table I except that concentration of ceramide dihexoside was varied K_m for ceramide dihexoside = $2.5 \cdot 10^{-4}$ M. Inhibition constant for ceramide dihexoside = $1.25 \cdot 10^{-5}$ M (see legend of Fig. 9 for the assumed inhibition model).

It is therefore difficult to relate the kinetic data in such a system to a biological context. The question as to whether the transferases are regulated in vivo by their own substrate concentration therefore must be left open.

It is known that in order to demonstrate conclusively that an exogenous lipid substrate is being utilised by a particle bound enzyme preparation it is not enough to show that it stimulates the reaction being studied. Incorporation of the exogenous substrate should be demonstrated unequivocally in order to offset the criticism that the added exogenous lipid is merely stimulating incorporation of endogenous substrate by alternate pathways. Labelled ceramide mono-hexoside and di-hexoside were therefore prepared by labelling NIL 2 cells with [^{14}C]palmitate and isolated by the 2-dimensional chromatography previously described. These glycolipids were then used as substrates in the assay system we have described. Using unlabelled UDP-galactose as the sugar donor under these conditions there was 0.15% conversion of labelled ceramide monohexoside to ceramide dihexoside and 0.05% conversion of labelled ceramide dihexoside to ceramide trihexoside. These are minimum estimates of the % conversion, since the cold endogenous glycolipid acceptors in the membranous enzyme fraction considerably dilute the specific activity of the added labelled acceptors.

Substrate specificities and effect of other glycolipids

Table II shows the effect of other glycolipids on the reaction rates. In addition to ceramide dihexoside from Miles Laboratories two other samples of ceramide dihexoside prepared from spleen and lung were also used for reaction II. The change in K_m values for transferase II when biologically isolated ceramide dihexoside was used instead of the synthetic ceramide dihexoside (Miles) was quite appreciable. With biological substrates this value was about 600 μM, while this was about 250 μM with the synthetic substrate using commercial

TABLE II

SUBSTRATE SPECIFICITIES AND THE EFFECT OF OTHER GLYCOLIPIDS ON REACTION RATES OF PARTICULATE ENZYME SYSTEMS CATALYSING THE SYNTHESIS OF CERAMIDE DIHEXOSIDE AND CERAMIDE TRIHEXOSIDE IN NIL-2 CELLS

The conditions of assay were as given in Table I. Reactions were stopped after one hour and the mixture was processed as described in the text. When a non-substrate glycolipid was present it was added along with the substrate in a chloroform/methanol solution and underwent the same emulsification procedure. The values reported are the arithmetic mean of separate experiments and the number of experiments are shown in parenthesis. A microsomal pellet prepared as described in the text was used as enzyme source. The rates given are those obtained before inhibition from kinetic plots.

Substrate glycolipid	Concentration in incubation mixture (μM)	Added non-substrate glycolipid	Glycolipid synthesized in pmol per mg protein per hour**		
			Concentration in incubation mixture (μM)	Ceramide dihexoside	Ceramide trihexoside
None	—	None	—	135	98(4)
Ceramide monohexoside (from Equine spleen)	75	None	—	780	121(4)
Ceramide monohexoside (from Bovine spleen)	75	None	—	768	128(4)
Ceramide monohexoside (from Porcine lung)	75	None	—	789	118(4)
Ceramide dihexoside (from Equine spleen)	50	None	—	228	458(6)
Ceramide dihexoside (from Bovine spleen)	50	None	—	210	492(6)
Ceramide dihexoside (chemically synthesized Miles laboratories)	50	None	—	228	578
Ceramide monohexoside (from Bovine spleen)	50	Ceramide dihexoside (from Miles laboratories)	50	908	697(3)
Ceramide monohexoside (from Bovine spleen)	50	Ceramide trihexoside (from Equine spleen)	50	883	187(13)*
Ceramide monohexoside (from Bovine spleen)	50	Ceramide tetrahexoside (from Equine spleen)	50	986	235(4)*
Ceramide monohexoside (from Bovine spleen)	50	Ceramide pentahexoside (from Equine spleen)	50	896	248(4)
Ceramide monohexoside (from Equine spleen)	50	Hematoside (from Equine spleen)	50	774	141(3)
Ceramide dihexoside (from Miles laboratories)	50	Ceramide trihexoside (from Equine spleen)	50	226	651(3)*
Ceramide dihexoside (from Miles laboratories)	50	Ceramide tetra saccharide (from Equine spleen)	50	217	714(4)*
Ceramide dihexoside (from Miles laboratories)	50	Ceramide pentahexoside (from Equine spleen)	50	236	810(4)
Ceramide dihexoside (from Miles laboratories)	50	Hematoside	50	215	785(4)

* The radioautographs showed strong labelling in the region of tetra- and penta glycosyl ceramides in these systems.

** Glycolipid synthesis is calculated from the specific activity of the donor sugar nucleotide assuming the stoichiometry of transfer as shown in Scheme I. Since it is obvious from the data presented that endogenous glycolipid participates in reactions studied in variable amounts more than one sugar could be labelled in biosynthesized ceramide trihexoside. Consequently an unknown correction factor will be involved between the number of moles of galactose incorporated and the number of molecules of glycolipid synthesized depending upon the degree of participation by endogenous substrates.

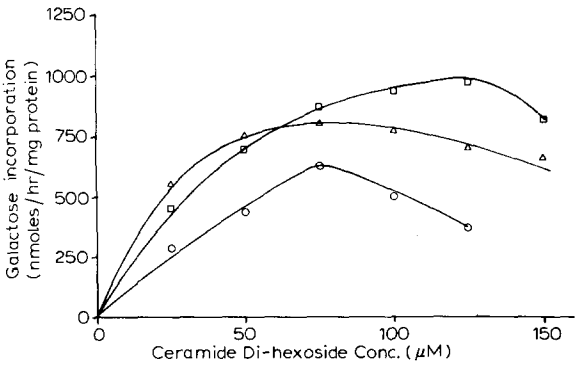


Fig. 10. Effect of ceramide dihexoside concentration on the rate of formation of ceramide trihexoside in the presence of added glycolipids. ○———○, ceramide dihexoside at indicated concentrations. □———□, rate of reaction with added exogenous ceramide trihexoside (50 μM) △———△, rate of reaction with added exogenous hematoside (50 μM). The conditions of assay were the same as in Table II.

cerebrosides from various sources as glycolipid substrates. Transferase I did not exhibit any such change in its kinetic constants. This behaviour of transferase II is noteworthy since the synthetic ceramide dihexoside differs from the biological compound only in a double bond in the sphingosine moiety.

The results also show that none of the other glycolipids tried can act as substrates for the reactions under study. However, as shown in Fig. 10 they consistently increased the observed rates of formation of both ceramide dihexoside and ceramide trihexoside when present in the assay mixture. It can be seen that the glycolipids tested shift all three kinetic constants, viz. the K_m, V and the inhibition constant. The most likely explanation is that these enzyme systems are sensitive to changes in the structure of their substrate micelles. Increasing the detergent concentration to keep the glycolipid/detergent ratio constant results in substantial inhibition of activity when the detergent concentration exceeds 4 mg/ml, presumably because of enzyme denaturation.

Apart from the stimulation of ceramide dihexoside and ceramide trihexoside synthesis which occurs at every concentration of the respective glycolipid substrate tied below its inhibition point, when added non-substrate glycolipid is present. Table II also shows that significant enhancement of endogenous synthesis occurs. This may be due to the endogenous glycolipid participating in the mixed micelle formations. An interesting observation is that trihexoside in reaction II and dihexoside in reaction I when present in the medium stimulated their own synthesis rather than showing any back reaction, and this emphasises the point that an in vitro assay system utilising micellar substrates can hardly be used to investigate regulatory processes like feedback control.

Inhibition by substrate analogues

Several studies have shown that the activities of glycoprotein glycosyl transferases obtained from a variety of tissues are affected by nucleotide phosphate. Both inhibitory and stimulatory effects have been described [19—22]. Considerable activation of glycolipid-glucosyl transferase has been shown to occur when phospholipids are included in the assay medium [23,24].

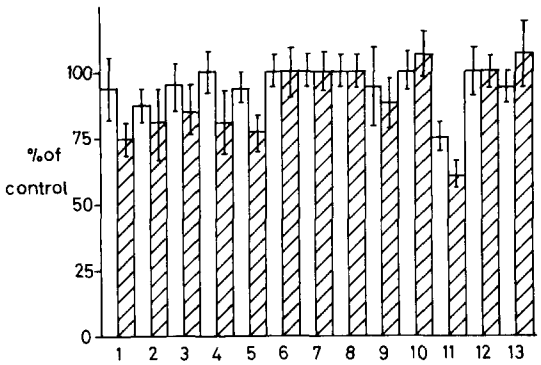


Fig. 11. Effect of nucleotides and phospholipids on ceramide dihexoside and ceramide trihexoside synthesis in NIL-2 cells. Conditions were the same as in Experimental Procedure. Phospholipids when present were dried along with the glycolipid substrate from a chloroform/methanol solution (2 : 1) in nitrogen. When nucleotides were present they were preincubated with enzyme particles for 15 min at 37°C. The crude microsomal pellet prepared as described in the experimental procedure was used as enzyme source. Reaction was stopped after one hour and the mixture was processed as described in the text. The data is expressed as % change in activity compared to controls. □, Transferase I; ▨, Transferase II. Phospholipid concentrations were 50 μM and nucleotide concentrations were 200 μM, when they were present in the incubation mixture. 1, Phosphatidyl inositol; 2, phosphatidyl choline; 3, phosphatidyl ethanolamine; 4, cardiolipin; 5, sphingomyelin; 6, control incubations (no additions); 7, GTP; 8, CTP; 9, UDP; 10, ADP; 11, ATP; 12, UTP; 13, cyclic 3′5′AMP.

The effect of a variety of phospholipids and ribonucleotide triphosphates were therefore tested to the transferases under study. Fig. 11 summarises the results obtained.

No consistent stimulatory effect was obtained with any of the compounds tried. Consistent inhibition was however obtained with ATP among the nucleotide triphosphates tried. The inhibition was pronounced only with transferase II under the conditions employed. The inhibition seems to be non-competitive because (a) K_m for UDP-galactose does not seem to be affected, (b) pre-incubation with UDP-galactose or substrate glycolipid has no effect on inhibited velocities. It is unlikely that an irreversible protein phosphorylation as a result of ATP treatment takes place because enzyme particles pre-incubated with ATP, (0.2 mM) for 30 min at 37°C, and dialysed for 3 h (3 changes of distilled water) gave uninhibited velocities comparable to that of a similarly treated control preparation without ATP. Presence or absence of Mg^{2+} at 0.5—2 mM did not effect the observed kinetic parameters in any significant way.

The effect of phospholipids on reaction rates as shown in Fig. 11 was unexpected because, most published data shows an activation of membrane bound enzyme systems with these micelles in an aqueous system. No activation was noticed even at concentrations much higher than those quoted in the figure.

Discussion

Previous studies on glycolipid metabolism in NIL2 cells showed that ceramide tri, tetra, and penta saccharides increase in concentration as cell population density increased [3—6]. Since the nature and quantity of each glycolipid

is probably controlled by the specificity and concentration of the corresponding glycosyl transferase, it was hoped that an understanding of the properties of these enzymes might lead to further clarification of glycolipid metabolism in these cells. A prior report [13] has described some properties of these enzymes, but did not deal with their kinetic behaviour. Our results show that the enzymes are susceptible to inhibition and activation by a variety of reagents. The fact that they are very sensitive to inhibition by their own substrates emphasizes the need to construct substrate-velocity curves in assays of enzyme levels in cell cultures. The data we have given shows that transferase II has an appreciably different K_m for a synthetic substrate than for substrates isolated from biological sources. This observation suggests that the enhanced synthesis of ceramide trihexoside by the dense cells may be due to subtle changes in the chemical structure of the enzyme substrate, so that a particular transferase would utilise a precursor pool of a particular chemical structure, when more than one such pool exists in the cell. This idea gains credence from studies on pig erythrocyte [25] glycolipids that show that palmitic, stearic and oleic acids are the principal fatty acids in the cerebrosides. However with increasing chain length of the oligosaccharide unit, the proportion of these acids drop to 1/9th of the original precursor cerebroside, the difference being made up by increasing the amount of behenic, lignoceric and nervonic acids, which were only trace compounds in the original cerebrosides. Assuming the accepted pathway one cannot reconcile these observations except by attributing a rigid specificity of various transferases toward their respective substrates discriminating them even when the difference is as subtle as the chain length of fatty acid, or when sphingosine instead of sphinganine is on the long chain base part of these molecules. Increased ceramide trihexoside synthesis therefore would result from a preferential utilisation of a ceramide dihexoside by tranferase II which is being made by the cell only when they are in contact and have attained a high density.

The following observations suggest that transferase I is different from transferase II in spite of their occurrence in the same particles, with similar pH optima:

(1) The two activities are clearly separated on a linear sucrose gradient. (see accompanying paper); (2) The inhibition by phospholipids, and ATP affects the two transferases differentially; (3) They are markedly different in their sensitivity to heat; (4) The ratio of the two activities that is obtained after the enzymes are released by detergent in a soluble form (i.e. not sedimented at $100 \cdot 10^3$ g for 1 h) differs appreciably from the original particulate fraction.

Chien et al. [26] have described the synthesis of a globoside-like glycolipid from tri-hexosyl ceramide in an in vitro system with an enzyme derived from embryonic chicken brain. Ishibashi et al. [27] have also synthesized globoside and Forssman hapten using Guinea-pig kidney and Yeung et al. [12] achieved a similar reaction with adrenal tumor cell material. In all these experiments the conditions employed for these syntheses were very similar to those we have described for enzymes I and II.

We have been unable to demonstrate the in vitro synthesis of tetra and penta glycosyl ceramides in NIL2 cells when ceramide trihexoside and globoside are given as glycolipid substrates and UDP-*N*-acetylgalactosamine as the nucleotide

sugar donor, using the reaction conditions found to be optimal for transferase I and II. On the other hand using UDP-galactose as sugar donor, two products were formed in the same system. The compound formed when ceramide trihexoside was used as glycolipid substrate had an R_F value identical to globoside in solvent system 65 : 25 : 4 (chloroform/methanol/water) and the compound formed when globoside was used as glycolipid acceptor had an R_F value identical to pentaglycosyl ceramide in the same solvent system. These reactions cannot be explained on the basis of known structures of tetra and penta glycosyl ceramides (Scheme I) except as a non-physiological specificity of a transferase present in the cell. More puzzling is the observation that under identical conditions the physiological reaction is not catalysed when correct substrates and cofactors are used. Stoffyn et al. [28] obtained the same result with material from rat tissues. They attribute their failure to synthesize globoside from ceramide trihexoside (prepared from erythrocytes and having a 1-4 linkage between two galactoses) using a rat tissue enzyme to a specificity of *N*-acetylgalactosaminyl transferase which would require a ceramide trihexoside molecule with a 1-3 linkage between the galactosyl units.

Such observations emphasize the need for using the stereochemically correct substrate glycolipid in in vitro synthesis. Less likely explanations would be that the in vitro synthesis of globoside and penta saccharide may be taking place as enzyme bound intermediates, or even as a one step synthesis by a di- or trinucleotide transfer from UDP-oligosaccharides as suggested by Roseman [9].

Attempts to demonstrate transferase II activity in transformed cells have failed. This may be due to a genetic block in enzyme synthesis or the presence of a non-dialysable particulate inhibitor which specifically inhibits the enzyme. Alternatively a catalytically defective enzyme protein may be a consequence of transformation.

Addendum

After the preparation of this manuscript a study of galactosyl transferase was reported in rat tissue [29]. The inhibition of reaction at higher substrate concentration was also observed in this study.

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