CELL MITOTIC CYCLE SYNTHESIS OF NIL HAMSTER GLYCOLIPIDS INCLUDING THE FORSSMAN ANTIGEN

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ABSTRACT

The synthesis of phospholipids and glycolipids during the cell mitotic cycle of an established hamster line, NIL, has been studied. Cells were synchronized with excess thymidine and mitotically harvested by shaking. Cells were radioactively labeled for 4 h with palmitate, glucosamine, or galactose. Lipids were analyzed by thin-layer chromatography. As cells progressed through the mitotic cycle, incorporation into phospholipids increased but the fraction represented by each remained constant. Similarly, ceramide monohexoside, dihexoside, and hematoside were labeled equally in all phases. Ceramide trihexoside and tetrahexoside were labeled only during G_1 and S.

Ceramide pentahexoside (the Forssman antigen) shows density-dependent synthesis, accumulation, and reactivity. Ceramide pentahexoside was labeled during all phases of the mitotic cycle but the rate of incorporation decreased in S and G_2. The total amount of lipid assayed immunologically in cell extracts gradually increased.

Exposure of the Forssman antigen in untreated or trypsin-treated cells was studied using binding of chemically labeled antiForssman antiserum. The amount of antigen detected in trypsinized cells increased during G_1 and early S but then remained constant. Mitotic cells exposed all detectable antigen. As cells progressed through the mitotic cycle, a large fraction of the Forssman antigen became cryptic.

INTRODUCTION

A number of laboratories have studied the metabolism of glycolipids in normal and transformed cells (1–18). In general, it has been proposed that transformed cells are blocked in the synthesis of various complex glycolipids. However, in some cell lines, the glycolipid pattern of transformed cells resembles the pattern observed in rapidly growing normal cells (2, 12). We were interested in complementing the study of the metabolism of glycolipids during the growth cycle (15) with a study of their synthesis during the mitotic cycle.

Previous studies on the synthesis of membranes during the mitotic cycle are conflicting. Some of the conflicts may be due to a loss of synchrony, to perturbations caused by the method of synchronization, to variations among cells in the composi-

tion of the membranes, or to variations in the different membranes within a cell. Most experiments have employed incorporation of radioactively labeled precursors as a probe for synthesis without any attempt to measure possible turnover, conversion of label, pool sizes, or transport rates. This is especially important in light of recent experiments showing that the transport of thymidine, uridine, and an amino acid analog varies during the mitotic cycle (19). Further, although membrane fractions were isolated in some studies (20–22), no attempts have been made to purify individual components. The observations can be summarized as follows: (*a*) synthesis of membrane components throughout the mitotic cycle paralleling protein increases (21, 23, 24); (*b*) preferential synthesis of all components during one phase (20, 22, 25); or (*c*) preferential synthesis of different components during different phases of the mitotic cycle (26–29).

In contrast to these results, studies of the cellular surface architecture during the mitotic cycle have been consistent. Antigenic "sites" which are unreactive during S become reactive during mitosis (30–37). These sites include the fluorescent wheat germ agglutination binding site (31, 32), H-2 antigens (33, 36), Moloney virus-induced surface antigens (33, 35), H blood group antigen (30, 37), and surface antigens of a murine lymphoma (34). Often, increased reactivity is also observed in G_2 (36) and G_1 (33–37), but it is not clear whether this reflects a unique state of surface architecture present in G_1 or G_2, or an approach to or recovery from the mitotic state of the surface. It might also result from a contaminating population of mitotic cells. At mitosis, cells (*a*) are more susceptible to virus-induced fusion (38), (*b*) release half the surface-bound heparan sulfate (39), (*c*) have increased electrophoretic mobility (40), and (*d*) permanently expose newly synthesized glycoproteins (41–43).

Recently, the structure of the Forssman antigen was clarified (44). It is one of the neutral glycolipids present in the NIL hamster line, and the reactivity (45), synthesis (4, 6, 12–16), and quantity (6) of this antigen have been found to vary with cell density. In many ways the Forssman antigen is similar to the lectin agglutination sites which have been extensively studied (32, 46, 47). Like lectin agglutination sites, this antigen is more readily detected in virally transformed cells (48–52) or upon trypsin treatment of normal cells (51, 52). Since the wheat germ lectin binding site is one of the determinants exposed during the mitotic cycle (31), we were interested in the behavior of the Forssman antigen during the mitotic cycle.

MATERIALS AND METHODS

Synchronization

Clone 2C1 of the NIL hamster line was isolated and characterized in this laboratory (13), and was used 6–10 passages after clonal isolation. Cells were grown in Dulbecco's modification of Eagle's minimal essential medium containing four times the concentration of amino acids and vitamins in Dulbecco's, plus 50 μg/ml streptomycin and 75 U/ml penicillin (MEM-4×), (Grand Island Biological Co., Grand Island, N. Y.), as described in Sakiyama et al. (13).

Cells were synchronized with a double thymidine block (53), or with a double thymidine block plus a mitotic collection (54) with Colcemid (CIBA Pharmaceutical Co., Summit, N. J.) as follows. In the double thymidine block procedure, cells were treated with 3 mM thymidine (A grade, Calbiochem, San Diego, Calif.), for 8–10 h, washed twice with serum-free medium, incubated with 0.5 mM 2′-deoxycytidine-HCl (grade II, Sigma Chemical Co., St. Louis, Mo.), and no thymidine for 6–8 h, washed, blocked with thymidine for 8 h, washed, and released with deoxycytidine. This procedure was employed for the experiments mentioned in Figs. 1, 2 A, 3 B, 4 B, 5 (right third), and Tables II and III (right third). In order to obtain synchronized cells during G_1, cells released from a double thymidine block were mitotically harvested in Colcemid as follows. At 5 h after release from thymidine, cells were washed and incubated for 2 h in 15 ml of Joklik-modified minimal essential medium for spinner culture (Grand Island Biological Co.), plus four times the concentration of amino acids and vitamins in Dulbecco's, 10% fetal calf serum (Microbiological Associates, Inc., Bethesda, Md.), and 0.3 μg/ml Colcemid. Recovery from Colcemid arrest was reversible as judged by normal plating efficiency upon removal. Mitotic cells were harvested by shaking glass Blake bottles (200 cm^2). The bottle was held in a horizontal position and rocked manually from side to side 40 times. Selective detachment of refractile cells was monitored by phase-contrast light microscopy. Medium was harvested by decanting. Cells were centrifuged at 150 *g* for 5 min. Between 2 and 7 × 10^7 cells were obtained from 15 bottles (20–60% yield). The yield depended upon the density of cells in the Blake bottle. Cultures of sparse cells ($< 1.5 \times 10^4$ cells/cm^2) gave a higher percentage yield than dense cultures ($> 1.5 \times 10^4$ cells/cm^2). The mitotic index was assayed by swelling in hypotonic buffer and scoring the presence or absence of nuclear envelope (55). Cells were >95% viable as judged by trypan blue exclusion. This procedure was employed for the experi-

ments mentioned in Figs. 2 B, 3 A, 4 A, 5 (left two-thirds), 6–8, and Tables II and III (left two-thirds), IV, V, and VII.

Radiolabeling

Radioactively labeled compounds were obtained from New England Nuclear, Boston, Mass. To measure DNA synthesis, cells on cover slips were pulsed for 1 h with [*methyl*-^{3}H]thymidine (51 Ci/mmol, 10 μCi/ml for thymidine-released cells; 6.7 Ci/mmol, 1 μCi/ml for mitotically collected cells), washed by dipping in solution A (phosphate-buffered saline without Ca^{++} or Mg^{++} [60]), and precipitated by immersion into 100% methanol. Cover slips were broken and inserted into counting fluid and counted in a Beckman liquid scintillation counter, LS-230 (Beckman Instruments, Inc., Fullerton, Calif.).

[1-^{14}C]Palmitic acid (59 mCi/mmol) medium was prepared according to Robbins and Macpherson (12). Cells were labeled in MEM-4× for 4 h with 1 μCi/ml [1-^{14}C]palmitate, 2.5 μCi/ml [1-^{14}C]glucosamine-HCl (58 mCi/mmol), or 2.5 μCi/ml [U-^{14}C]galactose (63.5 mCi/mmol), washed five times with solution A, and scraped into solution A or 100% methanol.

Extraction of Lipids and Analysis

Cells harvested in solution A were centrifuged and drained. Cells scraped in methanol were dried under nitrogen. No differences in lipid recovery were observed between methods but recovery of protein was better when solution A was used to harvest cells. Dry pellets were extracted three times with 1 ml chloroform-methanol (2:1). Aliquots were counted and the rest of the solution was dried under nitrogen.

Lipid extracts were applied to activated (1 h, 110°C), precoated thin-layer chromatographic plates (Silplate F-22, Brinkmann Instruments, Inc., Westbury, N. Y., or Merck silica gel F-254, Merck Chemical U.S.A., Rahway, N. J.). Plates were run in ascending chromatography according to Gray (56) as modified by Robbins and Macpherson (12). The first solvent system was chloroform-methanol-water (65:25:4). The second solvent system was developed perpendicular to the first and consisted of tetrahydrofuran-dimethoxymethane-methanol-water (60:36:30:6). Plates were dried overnight over desiccant between first and second directions. X-ray films (RP-R2, Eastman Kodak Co., Rochester, N. Y.) were exposed to the plates and radioactive spots were scraped and counted without prior elution. The silica gel did not result in quenching of ^{14}C counts. Palmitate data are expressed as counts in a lipid divided by counts in phospholipids plus glycolipids × 100. Protein was determined according to Lowry et al. (57) on total cells (Fig. 3, 6 A) or on lipid-extracted cells (Fig. 6 B and Tables IV, V) using bovine serum albumin as a standard.

Inhibition of Hemolysis by Cell Extracts

Lipid cell extracts were dried under nitrogen. 0.1 ml of 100% methanol containing 1 mg/ml azolecithin (purified soya phosphatides, Associated Concentrates, Woodside, N. Y.) was added and mixed vigorously. 0.9 ml of fresh diluent buffer (0.14 M NaCl, 0.01 M tris[hydroxymethyl]aminomethane, 5 mM $MgSO_4$, 1.5 mM $CaCl_2$, pH 7.4, 1 mg/ml gelatin) was added and gently mixed by pipetting. Twofold serial dilutions were made. To the 0.5-ml test sample, 0.1 ml commercial antisheep hemolysin (Grand Island Biological Co.), diluted 1:5,000 in fresh diluent buffer, was added and the sample was incubated 90 min at 37°C. Then 0.5 ml washed 1% sheep red blood cells (Connecticut Valley Biological Supply Co., Southampton, Mass. or Scott Laboratories, Inc., Fiskeville, R. I.), plus guinea pig complement (1:140 dilution, Grand Island Biological Co.), was added and incubated 75 min at 37°C. The test tubes were chilled in ice and centrifuged. Supernates were read with a Zeiss PM QII 15,000 spectrophotometer (Carl Zeiss, Inc., New York), at 550 nm to measure hemoglobin release. Standards containing 0–0.5 ml hemolysin, diluted 1:16,000, and crude antigen extracts were included each time as standards. Sheep red blood cells were between 1 and 4 wk old for all experiments. No lysis occurred without antibody, and anticomplement activity was not observed.

Binding of Radioactive Antibody to Whole Cells

Rabbit antiserum prepared against purified Forssman glycolipid was kindly supplied by Dr. S. Hakomori (45). An equal volume of saturated $(NH_4)_2SO_4$ (wt/vol) was added to the antiserum. The washed precipitate was dialyzed and labeled with [^{14}C]formaldehyde (New England Nuclear, 59 mCi/mmol) according to Rice and Means (58). Binding studies were done with intact monolayers preincubated with 0.3 mg/ml bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill.) and 0.3 mg/ml rabbit gamma globulin (kindly supplied by Dr. L. Steiner, Massachusetts Institute of Technology, Cambridge, Mass.) at 37°C for 15 min. Radioactive antiserum (12.5 μg, 1,000 cpm/μg) was added and cells were incubated on ice for 10 min. Binding was complete by 10 min (61). Plates were washed with solution A, scraped, and counted. Binding studies with trypsinized cells were carrried out similarly except for the following modifications. Monolayers were washed with solution A and treated with 25 μg/ml trypsin (crystallized three times, Worthington Biochemical Corp., Freehold, N. J.), for 10 min at 37°C. At this time, all the cells had detached from the plate and an equal volume of soybean trypsin inhibitor solution (Worthington, 50 μg/ml) was added. The cell suspension was centrifuged, washed, and resuspended in solution A. After preincubation, cells

were incubated with antiserum as above. After 10 min, incubation mixtures were diluted twofold and cells were centrifuged, washed, and counted. Controls with no cells or clones without antigenic reactivity in the hemolysis assay were at background level in the binding assay. The binding is linear with antiserum concentration (61).

RESULTS

Synchronization

4 h after release from the second thymidine block, the cell number began to increase (Fig. 1). Between 85 and 100% of the cells divided within the next 4 h. 1-h pulses with radioactive thymidine showed that incorporation of label into alcohol-precipitable material was high after release from thymidine block (Fig. 2 A). By 4 h after the release when mitotic cells began to appear, the level of incorporation into precipitable material was lower. This low level was maintained for at least 5 h when the experiment was terminated. These results suggest that cells released from a double thymidine block are in S phase but not at the G_1-S border. Instead, these cells exhibit shorter S and G_2 phases than expected, as observed by Bostock et al. (59). Decreasing the length of excess thymidine block from 10 to 8 h resulted in a slightly longer lag before cell division (data not shown). The results obtained by exposing cells which were released from a double thymidine block to [^{14}C]palmitic

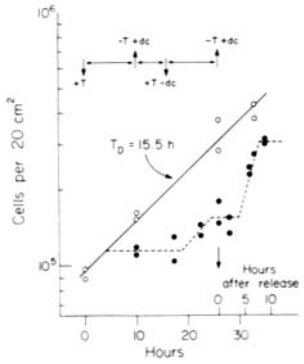

FIGURE 1 Cells were seeded onto 20-cm² petri dishes. At the times indicated, the cell monolayer was washed twice with serum-free medium, and fresh medium containing thymidine or deoxycytidine was added. Control cells were washed and fed with fresh medium at the same time. The arrow indicates the beginning of the time-scale for the synchronized population. T_D = generation time. o——o, control cells. o---o, synchronized cells.

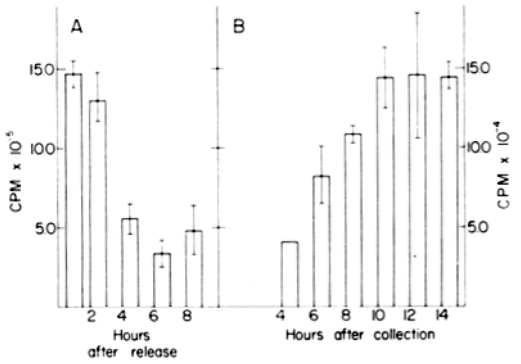

FIGURE 2 Cells on cover slips were pulsed for 1 h with [*methyl*-^{3}H]thymidine, either 10 μCi/ml A, or 1 μCi/ml B, washed in solution A, and methanol precipitated. Cover slips were counted in a liquid scintillation counting system. The bars indicate variation in duplicate plates. (A) Cells were blocked twice with excess thymidine as detailed in Materials and Methods. Zero time represents release from the second block. (B) Mitotic cells were collected by a 2-h exposure to Colcemid 5 h after release from the second thymidine block. Zero time represents release from Colcemid arrest.

acid are shown in the right third of Tables II and III. The 0–4-h period probably represents cells in all parts of S, while the 4–8-h period undoubtedly contains a mixture of cells in all phases.

In an attempt to obtain cells synchronized in G_1 and the beginning of S, cultures released from a double thymidine block were mitotically collected in Colcemid. Cultures released from the double block did not yield enough mitotic cells for our biochemical studies unless Colcemid was added to prevent exit from mitosis. Colcemid collection for 2 h during the sharp cell-doubling period shown in Fig. 1 yielded 10–40 million cells. Routinely >95% and often ≥98% mitotic cells were obtained. These cells attached within 0.5 h and were completely flattened by 1 h. Pulses with [^{3}H]thymidine (Fig. 2 B) suggested that S phase began at 4–6 h after mitosis. However, cells were still in S phase 15 h after mitosis. Until 16 h after mitosis, no mitotic cells were observed. After 16 h cell number began gradually to increase (data not shown). The doubling time of NIL 2Cl was 15–16 h (Fig. 1). Protein content increased gradually during the mitotic cycle (Fig. 3). During the first 3 h after collection, no more than 10% of the cells were in S phase. In the next hour, 25% of the cells had entered S. By 8 h after collection, 75% of the cells

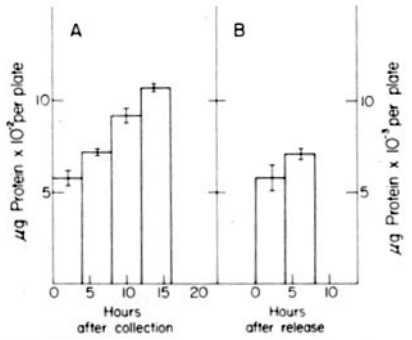

FIGURE 3 Protein was determined on whole cells by the method of Lowry et al. (57). Bars indicate variation between duplicate plates. (A) Mitotic cells were collected by Colcemid arrest 5 h after release from the second thymidine block. Zero time represents release from Colcemid arrest. (B) Cells were blocked twice with excess thymidine. Zero time represents release from thymidine block.

TABLE I

*Glycolipid Nomenclature**

Ceramide-glc Ceramide-gal	GL-1
Ceramide-glc-gal	GL-2
Ceramide-glc-gal-gal	GL-3
Ceramide-glc-gal-gal-galNAc	GL-4
Ceramide-glc-gal-gal-galNAc-galNAc	GL-5
Ceramide-glc-gal-NANA Ceramide-glc-gal-NGNA	G_{M_3}

*Abbreviations: glc, D-glucose; gal, D-galactose; galNAc, *N*-acetyl-D-galactosamine; NANA, *N*-acetyl-neuraminic acid; NGNA, *N*-glycolyl-neuraminic acid.

were in S phase. By 10 h, all of the cells were incorporating [^{3}H]thymidine (Fig. 2, continuous labeling data not shown). Although no increase in cell number was observed before 16 h, the gradual cell doubling seen afterwards suggests a loss of synchrony. The period between 12 and 16 h after Colcemid release consists of cells in S and G_2 phases, but contains few M and G_1 cells because no cell doubling or mitotic cells were seen.

Lipid Analysis

The structure and nomenclature of the glycolipids studied are given in Table I. Unidentified spots

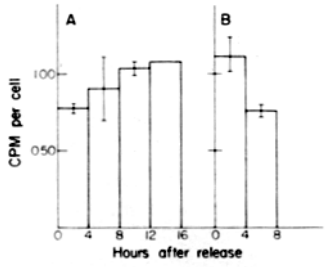

FIGURE 4 Cells were labeled with 1 μCi/ml radioactive palmitate for 4 h, washed, and scraped. Cells were extracted with chloroform-methanol (2:1) and run on thin-layer chromatography as detailed in Materials and Methods. The graph represents a summation of counts per minute incorporated into all the phospholipids and glycolipids divided by the number of cells extracted. Bars represent variations observed between three separate experiments. (A) Cells were mitotically harvested 5 h after release from a double thymidine block. Zero time represents release from Colcemid block. (B) Cells released from the double thymidine block. Zero time represents the release from the second block.

were preliminarily characterized by incorporation of [^{32}P]phosphoric acid, [^{14}C]palmitate, and [^{14}C]sugars but were not further examined.

Incorporation of palmitate into total phospholipids and glycolipids increased on a per cell basis as the cell progressed through the cycle (Fig. 4), but considerable labeling occurred in G_1 (Fig. 4 A). The decrease in incorporation per cell seen in Fig. 4 B is due to the doubling in cell number by 8 h. The percentage of counts in several phospholipid spots in a 4-h pulse with palmitate, averaged over three experiments, is shown in Table II. In the chromatographic system used, sphingomyelin (SM) and phosphatidylcholine (PC) did not separate well. SM + PC represented 75–80% of the palmitate incorporation, phosphatidylethanolamine represented 8%, and phosphatidylinositol was 5%. Longer labeling periods gave similar distributions (reference 13, and B. A. Wolf, unpublished observations). Each phospholipid incorporated palmitate at the same rate during all phases of the cell cycle.

In Table III, the labeling pattern of the glycolipids is shown. GL-1, GL-2, and G_{M_3} showed no preferential labeling during any phase of the cell cycle. On the other hand, GL-3 was labeled during G_1 and GL-4 was labeled during G_1 and early S. Fig. 5 A shows the variation among experiments in the fraction of the radioactivity in G_{M_3}. There was

no significant variation in the degree of labeling during any time period. Fig. 5 B shows the same data for GL-4. Within a given experiment, the incorporation into GL-4 was always greatest in G_1 and early S, and decreased or was not observed in late S and G_2. GL-3 represented so few counts that it was difficult to find in most autoradiograms. One experiment done with mitotically collected cells was exposed to X-ray films for 2 mo. In the first two labeling periods, GL-3 was observed but no radioactive spot could be found in the cells in the last two periods even though comparable total counts were spotted. Since background was <0.01, GL-3 in the 8–12- and 12–16-h periods must be ≤ 0.01. In contrast to the other lipids, the pattern of labeling of GL-5 was quite variable and

TABLE II

*Incorporation of [^{14}C] Palmitate into Phospholipids**

	Mitotic release					Double T release	
	M — G_1 — S — G_2 — M					S — G_2 — M	
Labeling times	0–4	4–8	8–12	12–16	16–19	0–4	4–8
Spot							
Sphingomyelin + Phosphatidylcholine	81.8	78.8	75.3	77.9	76.6	79.1	76.0
Phosphatidylethanolamine	6.0	8.4	9.6	9.0	8.7	7.8	7.8
Phosphatidylinositol	3.4	3.5	4.8	3.7	5.2	4.1	5.3

Cells were labeled with 1 μCi/ml radioactive palmitate for 4 h and the lipids were isolated and analyzed by thin-layer chromatography as detailed in Materials and Methods. On the left side is shown the average of three experiments performed on cells released from a mitotic collection with Colcemid after a double thymidine block. On the right third is shown the average of three experiments performed on cells released after a double thymidine block. The total counts per minute varied between experiments from 6.5×10^5 to 3.0×10^6 cpm/4-h period. Background was <0.01% of total.

* Numbers are expressed as incorporation into phospholipid divided by incorporation into phospholipids plus glycolipids $\times$ 100. Numbers are averages of three separate experiments which differed by no more than 10%.

TABLE III

*Incorporation of [^{14}C]Palmitate into Glycolipids**

	Mitotic release					Double T release	
	M — G_1 — S — G_2 — M					S — G_2 — M	
Labeling times	0–4	4–8	8–12	12–16	16–19	0–4	4–8
Spot							
GL-1‡	1.70	1.50	1.20	1.30	0.94	1.70	1.90
GL-2§	0.03	0.04	0.03	0.03	0.03	0.04	0.02
GL-3‖	0.08	0.06	≤0.01	≤0.01	ND	ND	ND
GL-4¶	0.37	0.45	0.31	0.07	0.16	0.06	0.06
GL-5**	0.47	0.61	0.58	0.67	0.41	0.28	0.22
G_{M3} ‡‡	1.05	1.20	1.18	1.15	1.00	1.06	1.20

See legend to Table II.

* Numbers are expressed as incorporation into glycolipid divided by incorporation into phospholipids plus glycolipids $\times$ 100. Numbers are averaged over three separate experiments (except for GL-3).

‡ $\pm$0.4.

§ $\pm$0.02.

‖ Detected in only one experiment.

¶ Variations between experiments were sometimes large but consistent within a given experiment. See Fig. 5.

** $\pm$0.2.

‡‡ See Fig. 5.

ND = not determined.

no systematic pattern was observed either in individual experiments or in the average of several experiments.

In order to clarify the labeling pattern of GL-5 and to confirm the results with the other glycolipids, synchronized cells were labeled with [^{14}C]glucosamine. Several laboratories have found that glucosamine is incorporated into acid-insoluble material as *N*-acetylglucosamine, *N*-acetylgalactosamine, and *N*-acetyl- (or glycolyl)-neuraminic acid (62–65). In contrast to the palmitate label, the total incorporation of glucosamine into acid-precipitable material was highest in G_1 (see Fig. 6 A). Incorporation into the glycolipids represented between 1 and 2% of the total at all times (Fig. 6 B). The glycolipids probably were labeled only in *N*-acetylgalactosamine or neuraminic acid (see Table I) since metabolism of label to other lipids (GL-1, GL-2, and phospholipids) was not observed, suggesting that radioactive glucosamine did not enter the carbon pool. As shown in Table IV, approximately equal incorporation of glucosamine into G_{M_3} occurred during all labeling periods, except for 8–12 h. Incorporation into GL-4 was slightly higher in G_1 and early S than in late S and G_2. GL-5 was clearly labeled at all phases but incorporation decreased in late S and G_2.

The incorporation of radioactive galactose in 4-h periods during the mitotic cycle is shown in Table V. It has been found that >85% of the label remains as radioactive galactose in acid-insoluble material (64). When radioactive galactose is metabolized it is found as glucose and amino acids

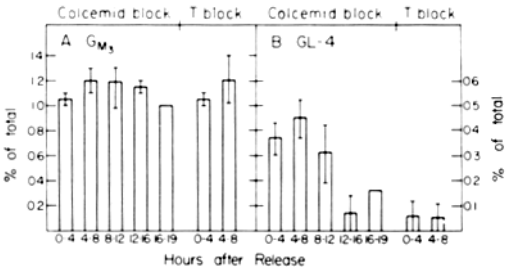

FIGURE 5 Cells were labeled with 1 μCi/ml radioactive palmitate for 4 h and the lipids separated as detailed in Materials and Methods. On the left of the graphs are shown glycolipids isolated from cells released from Colcemid collection after a double thymidine block. On the right of the graphs are shown glycolipids isolated from cells released from a second thymidine block. The bars represent variations between three separate experiments. (A) G_{M_3}. (B) GL-4.

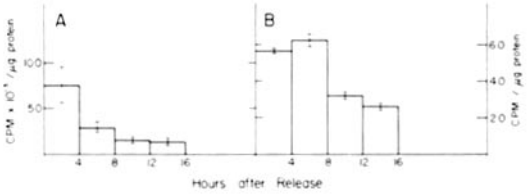

FIGURE 6 Cells released from a Colcemid collection after a double thymidine block were labeled for 4 h with 2.5 μCi/ml glucosamine-HCl. After washing with solution A, cells were scraped. Aliquots were precipitated with 5% cold trichloroacetic acid, filtered, and counted, (A). The remainder was extracted with chloroform-methanol (2:1) and the lipids were separated as detailed in Materials and Methods. The total counts per minute incorporated into the glycolipids were graphed, (B). The bars represent variation in duplicates. Protein was determined on whole cells (A), or lipid-extracted cells, (B). Zero time was at release from Colcemid arrest.

TABLE IV

Incorporation of [^{14}C]*Glucosamine into Glycolipids**

Labeling times	0–4	4–8	8–12	12–16
Spot				
GL-4	0.52 ± 0.2‡	0.62 ± 0.4	0.22 ± 0.07	0.17 ± 0.06§
GL-5	4.7 ± 0.4	5.3 ± 0.1	2.8 ± 0.1	2.1 ± 0.1
G_{M_3}	0.48 ± 0.04	0.35 ± 0.07	0.16 ± 0.01	0.44 ± 0.08

Cells were mitotically harvested after release from double thymidine block, as detailed in Materials and Methods, and labeled for 4 h with 2.5 μCi/ml [^{14}C]glucosamine-HCl. Zero time represents release from Colcemid arrest. Labeling medium contained 1,000 × molar excess of glucose. Lipids were analyzed as described in Materials and Methods. Protein was determined on lipid-extracted cells.

* Numbers are expressed as counts per minute incorporated into glycolipids per microgram protein.

‡ Numbers are the average of duplicates ± variation in duplicates.

§ 3 × background levels.

TABLE V

*Incorporation of [^{14}C]Galactose into Glycolipids**

Labeling times	0–4	4–8	8–12	12–16
Spot				
GL-1	2.9 ± 0.3‡	2.5 ± 0.15	2.4 ± 0.3	1.5 ± 0.3
GL-2	0.40 ± 0.05	0.19 ± 0.05	0.24 ± 0.05	0.15 ± 0.04§
GL-3	1.8 ± 0.3	0.92 ± 0.2	0.62 ± 0.3	0.41 ± 0.15
GL-4	1.3 ± 0.3	0.71 ± 0.3	0.55 ± 0.3	0.35 ± 0.04
GL-5	10 ± 2	5.7 ± 1	6.2 ± 2	3.8 ± 0.05
G_{M_3}	9.3 ± 2	5.4 ± 1.5	8.0 ± 0.8	5.6 ± 1

Cells were mitotically harvested after release from double thymidine block, as detailed in Materials and Methods, and labeled for 4 h with 2.5 μCi/ml [^{14}C]galactose. Zero time represents release from Colcemid arrest. Labeling medium contained 1,000 × molar excess of glucose and 10–100 × molar excess of amino acids. Lipids were analyzed as described in Materials and Methods. Protein was determined on lipid-extracted cells.

* Numbers are expressed as counts per minute incorporated into glycolipids per microgram protein.

‡ Numbers are the average of duplicates ± variation in duplicates.

§ 3 × background.

(64, 65). Although all the glycolipids were labeled during the 4-h exposure to radioactive galactose, no label was found in the phospholipids which represent the majority of lipids in the cell. The lack of conversion of galactose label is probably a result of the excess of glucose (25 mM) and amino acids 0.3–3 mM) over galactose (0.03 mM) in the labeling medium. The amount of label incorporated into GL-1, GL-2, and G_{M_3} varied no more than twofold during all labeling periods. GL-3 was labeled more heavily in G_1 and early S phases. GL-4 was labeled more heavily in G_1 and S phases. GL-5 incorporated galactose during the whole mitotic cycle but incorporation decreased in S and G_2 phases. The glucosamine and galactose incorporation data are consistent with the palmitate incorporation (see Fig. 7).

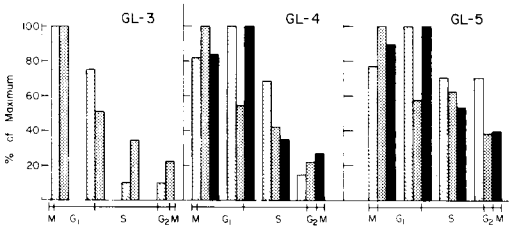

FIGURE 7 Cells were synchronized by a double thymidine block or by mitotic collection from monolayer after a double thymidine block. Cells were labeled for 4 h with 1 μCi/ml palmitate, 2.5 μCi/ml galactose, or 2.5 μCi/ml glucosamine-HCl, and lipids analyzed as described in Materials and Methods. The cross-hatched bars represent incorporation of palmitate. The dotted bars represent incorporation of galactose. The filled bars represent incorporation of glucosamine. The divisions of the mitotic cycle are approximate and are included to facilitate comparison.

Immunology

Since one of these glycolipids has been identified as the Forssman antigen (44), we were interested in the immunological reactivity of this lipid. An assay was devised based on the lysis of sheep red blood cells by hemolysis. This assay is highly specific and also highly sensitive, detecting picomoles of lipid antigen (61). Only GL-5 inhibited hemolysis (Table VI). Daily variations, perhaps due to the state and batch of red blood cells, necessitated the inclusion of both hemolysin and antigen standards.

In Table VII the total amount of antigen per cell in lipid extracts averaged over four determinations is shown. The amount of lipid antigen gradually increased two- to threefold during the mitotic cycle. Because of the large variations, these results should be interpreted cautiously but are consistent with synthesis of GL-5 throughout the mitotic cycle.

In Fig. 8, the binding of radioactive antiForssman antiserum to cells before and after trypsin treatment is shown. The amount of antiserum bound to trypsinized cells increased gradually until 8 h after mitosis. No additional binding was observed when mitotic cells were trypsinized. As the cells progressed through the mitotic cycle, the proportion of antigen exposed by trypsin treatment increased until 8 h after mitosis. Similar measure-

TABLE VI

Quantitation of GL-5 by the Hemolysin Assay

Glycolipid	Dilution^{-1} of sample giving 50% inhibition of hemolysis
NIL 2Cl cell extract	80,000
GL-5	46,000
GL-4	15
GL-3	<8
GL-2	<8
GL-1	<8
G_{M_3}	<8

Lipids were extracted with chloroform-methanol (2:1) from 2.4×10^7 cells and separated on thin layer chromatography, as detailed in Materials and Methods. The lipids were eluted from the silica gel with 100% methanol and rerun in one dimension in the first solvent. Eluted lipids were diluted serially and assayed for ability to inhibit hemolysis as detailed in Materials and Methods.

TABLE VII

Total Forssman Antigen in Extracts of Cells during the Cell Cycle

Time after mitotic collection	Antigen
h	*pmol/cell*
0	0.086 ± 0.023
4	0.11 ± 0.005
8	0.13 ± 0.051
11	0.13 ± 0.062
14	0.21 ± 0.023

Cells were harvested from Colcemid after release from a double thymidine block, as described in Materials and Methods. Zero time represents release from Colcemid arrest. Cells were harvested at the times indicated and lipid was extracted. Samples were stored at −20°C until assayed. Hemolysis was performed as detailed in Materials and Methods. 50% inhibition of hemolysis is achieved with 5.7 pmol of GL-5 (61).

ments have shown that 60–70% of the antigen detected in trypsinized growing cells and 70–95% of antigen in trypsinized confluent cells is unavailable to antiserum before trypsin treatment (45, 61).

DISCUSSION

We have attempted to obtain populations of highly synchronous cells. In order to study the glycolipids which are present at very small levels (<5%), large numbers of cells were necessary. For this reason

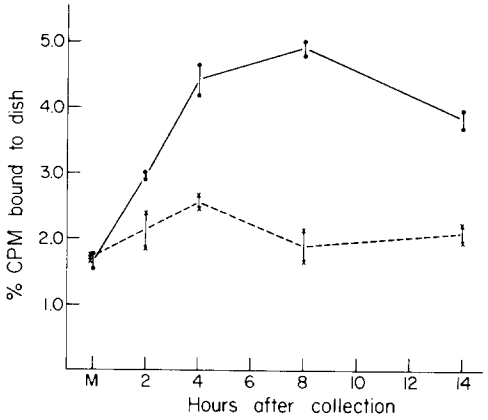

FIGURE 8 Radiolabeled antiForssman antiserum (12.5 μg, 1,000 cpm/μg) was added to untreated or trypsin-treated (10 min, 25 μg/ml) monolayers of cells mitotically harvested in Colcemid after release from a double thymidine block. Bars or linked X's indicate variation between duplicates. Zero time is at release from Colcemid arrest. Background <0.3%. ○——○, trypsin treated. ✕---✕, untreated.

we first employed a double block of excess thymidine (53) and modified the technique to our NIL clone. Initial experiments showed that 2–3 mM thymidine arrested cell division and was completely reversible, resulting in cell doubling by 10 h after release (Fig. 1 and reference 61). Although cultures synchronized by the double excess thymidine procedure were enriched for S-phase cells, shortening of the S + G_2 phases was observed and the moderate degree of synchrony deteriorated by 8 h. In an effort to regain a synchronized culture, Colcemid was added to arrest exit from mitosis. The level of Colcemid used was also reversible as long as exposure was brief. Prolonged treatment with Colcemid led to loss of viability. However, by a 2-h treatment with Colcemid, 10–40 million cells with mitotic index of 95–99% were obtained. These cells attached to the substratum within a half hour and were completely flattened by 1 h after release. No incorporation of radioactive thymidine was observed until 4 h after release. By 8 h after release, 75% of the cells were in S. No mitotic cells were observed until after 16 h. In our hands, the mitotic harvest from monolayer cultures resulted in highly synchronous populations which were viable and retained synchrony for most of the generation time (15–16 h).

It is possible that the use of excess thymidine

and Colcemid may have introduced perturbations in macromolecular synthesis. It has been observed in HeLa cells that the continued presence of inhibitors of DNA synthesis, including thymidine, resulted in unbalanced growth (66), that is, synthesis of RNA and protein in the absence of DNA synthesis. After 48 h, protein and RNA content doubled, as did cell volume. However, Cohen and Studzinski did not release cells from the inhibitors to see whether cells remained viable during the prolonged block. From observations with our clone, we would expect that prolonged exposure to inhibitors of DNA synthesis would significantly reduce viability. Although such unbalanced growth may occur during the double block procedure of the NIL clone, we tried to minimize perturbations by blocking for as short a time as possible and by using a low concentration of thymidine. The viability of the NIL cells subjected to a double thymidine block remained high, as evidenced by the high yield of cell doubling, 85–100%. The concentration of thymidine used resulted in an incomplete block to DNA synthesis, as evidenced by the shortened S + G_2 phase, probably due to slow movement of cells through S phase (59). Cohen and Studzinski did not observe that the unbalanced growth in the absence of DNA synthesis was harmful to the cell. We also do not have any evidence to suggest any harmful effects during the short exposure to thymidine. On the contrary, the cells released from a double thymidine block were highly viable, were able to divide, and, in the generation time after Colcemid collection, doubled their protein content (see Fig. 3 A). Although the use of inhibitors may have resulted in unbalanced growth during blockage of DNA synthesis, we have observed nothing to suggest that the cells were adversely effected. In addition, our results with total lipid synthesis agree with Pasternak and colleagues (21, 23, 24), who did not use inhibitors.

In agreement with Pasternak and colleagues (21, 23, 24), who studied a mouse mastoma, radioactivity was incorporated into the majority of lipids during all phases of the NIL cell cycle. This was true both for total lipids and for the majority of specific lipids. In contrast, Bosmann and Winston (26–28) have found synthesis of membrane components in S phase although glycolipids were made only in G_2. However, the lymphoma line studied by Bosmann has a short doubling time with almost no G_1 and G_2.

On the other hand, using similar labels, Gerner and collaborators observed incorporation during G_1 in human KB cells (20), while Nowakowski et al. reported a peak of fucose incorporation in G_2 (22). Plasma membranes were isolated by Gerner et al. and Nowakowski et al., but no attempt was made in any of these studies to fractionate the labeled components (protein or lipid). The different results could be due to variations in cell lipid composition, differences between plasma membranes and whole cells, different methods and degrees of synchrony, or variations in the synthesis of membranes among cell types.

Our studies on the fibroblast line, NIL 2Cl, cannot resolve all these conflicts, but may suggest reasons for the variations. Using highly synchronous populations, we have confirmed the observation that most of the lipids are labeled throughout the cell cycle. In addition, it is possible that a special class of components can be made only during a restricted part of the cycle. This special class could differ for each cell line or for different membrane fractions, i.e., plasma membrane vs. internal membrane.

The observation that the complex glycolipids are made during G_1 and early S shows an interesting parallel with studies performed during the growth cycle (4, 12–16). GL-3 was not significantly labeled until cells became confluent. GL-4 was labeled somewhat at low densities but incorporation increased at confluence. GL-5 was labeled at all densities but the highest rate of incorporation was in confluent cells. GL-3 and GL-4 were also labeled preferentially during G_1 and early S, while GL-5 was labeled during all phases of the cycle (although at a higher rate in G_1). Thus, the incorporation of label into GL-3 and GL-4 is restricted during the cell cycle and in growing cultures while the incorporation of label into GL-5 is less restricted.

We have used the term "rate of labeling" instead of "synthetic rate" because we cannot exclude the possibility of turnover. It was assumed that rapidly growing cells might be synthesizing membrane components at a maximal rate and that degradation would be minimal. By using radioactively labeled fatty acid and sugars, the possibility of inaccurate synthetic rates due to turnover was reduced. Degradation of the whole molecule, however, cannot be ruled out. The equal rate of labeling of some of the glycolipids throughout the cycle makes the possibility of artifacts due to pool sizes unlikely. We found no differences in the

neutral lipid fraction (which includes precursor pools) during the mitotic cycle.

The state of the cell surface during the cell cycle is another intriguing problem. As stated above, many different antigenic sites are "exposed" during mitosis (30–37). Although this may be a consequence of release of substances attached to the surface, e.g., heparan sulfate or other glycosylaminoglycans, some rearrangement of the existing architecture may also be involved. The mitotic cell seems to resemble a trypsinized cell, for example, in the spherical rounding of a flattened cell and in the more tenuous attachment to the substratum.

Alternatively, trypsin treatment or mitosis may result in greater mobility of membrane components. A third hypothesis is that mitosis and G_1 are the times in the cell cycle when antigenic sites are inserted into the membrane. Subsequently, they either become diluted in the bilayer as more phospholipid backbone is assembled, or covered by other membrane proteins or by extracellular material. In this context, it is interesting that Sander and Pardee (19) found decreased transport rates in mitosis and early G_1 which quickly returned to interphase-specific activity (rate per milligram protein) by late G_1 and S. We are currently investigating the release of extracellular material and the insertion of new material which is exposed to the surface. A fourth hypothesis is that mitotic cells are resistant to trypsin exposure of antigen. However, both the labeling data and immunological quantitation of GL-5 tend to exclude this possibility.

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Addendum

After this manuscript was submitted, Chatterjee et al. published a paper on the synthesis of glycolipids in synchronized KB cells (Chatterjee, S., C. C. Sweeley, and L. F. Velicier. 1973. *Biochem. Biophys. Res. Commun.* **54**:585). They observed a peak of incorporation of radioactively labeled galactose into acid-insoluble material during S and a peak of galactose incorporation into gangliosides and neutral glycolipids during G_2. However, they did not separate any of the labeled components, hence comparison with our results is difficult.

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