

SYNTHESIS OF GLYCOPROTEIN, GLYCOLIPID, PROTEIN, AND LIPID IN SYNCHRONIZED L5178Y CELLS

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ABSTRACT

Synthesis of four macromolecular classes found in membranes—glycoprotein, glycolipid, protein, and lipid—was measured as a function of time of the cell cycle in synchronized L5178Y cells. Incorporation of leucine, choline, fucose, glucosamine, or thymidine into the cells, protein, nucleic acid, or lipid was measured by pulse-labeling for 1/2 hr at 1/2 hr intervals after release from the mitotic block. The amount of protein, lipid, glycoprotein, or glycolipid released or secreted into the medium by the L5178Y cells was also measured as a function of time of the cell cycle. Cellular protein was found to be synthesized throughout the cell cycle, with the highest synthesis occurring in the S period; synthesis was depressed in the M period. Cellular glycoprotein was synthesized at approximately the same times as protein, except that the rates of glycoprotein synthesis in the S period relative to other periods were much greater than for protein. Secreted protein was synthesized throughout the cell cycle without any general pattern, except that secretion was elevated in the late S and G₂ periods. Secreted glycoprotein was similar to secreted protein. Cellular lipid and cellular glycolipid were synthesized almost exclusively in the G₂ and M periods; there was no synthesis in the G₁ and S periods. Release or secretion of glycolipid and lipid also occurred in the G₂ and M periods.

INTRODUCTION

Cellular membranes are known to contain both glycoproteins and glycolipids as normal (8, 28) and abnormal (17) constituents; most extracellular proteins occur as glycoproteins (13). Although the biosynthesis of glycoprotein and glycolipid in cell membranes has been studied (8) and many glycoprotein transferase enzymes (5, 10, 16, 21, 23, 30, 31) and glycolipid transferases (2-4, 24, 33) have been described in a wide variety of systems, little work has been done on the stage or stages within the cell cycle in which synthesis of glycolipids and glycoproteins occurs. Warren and Glick (32) studied the incorporation of D-glucose-¹⁴C and glucosamine-¹⁴C into L cell surface membranes

and found that growing and nongrowing cells synthesize approximately similar amounts of surface membrane and particulate material. The present study was undertaken to determine synthesis of protein, lipid, glycoprotein, and glycolipid in synchronized cells, since these macromolecules act as precursors of membrane formation.

Since fucose has been used in cell incorporation experiments (8, 19) and was found to be incorporated directly into macromolecules without epimerase transformation, it, as well as glucosamine, was used to measure glycoprotein and glycolipid synthesis in the present experiments.

Tritiated thymidine incorporation, routinely employed to measure DNA synthesis (25), was used in the present experiments, as was leucine incorporation to measure protein synthesis. Choline has been shown to be directly incorporated into lipid and has been suggested as a membrane marker (26); it was used as a measure of lipid synthesis.

Few studies have actually measured protein synthesis as a function of all stages of the cell cycle, but several studies have been published demonstrating synthesis in limited phases of the cell cycle. Johnson and Holland (18) and Prescott and Bender (27) indicated that protein synthesis is severely depressed during the M stage of the cell cycle. Conversely, Davies et al. (11) found that general protein and collagen syntheses occurred at approximately the same time in the cell cycle, in general at a constant rate, prior to release from FUDR block, after release from FUDR block, and prior to and during division. Gurley and Hardin (15) found that the synthesis of five histone fractions occurred mainly in the S phase in synchronized Chinese hamster cells. Measurement of activity of various enzymes as a function of phase of the cell cycle indicates that alkaline phosphatase is detected at a more or less constant rate over the entire cell cycle in synchronized HeLa cells (29), whereas glucose-6-phosphate dehydrogenase and lactate dehydrogenase exhibit three large peaks of activity 3, 7, and 10 hr postmitosis (20). Therefore, proteins as enzymes or structural components of the cell are synthesized in all phases of growth, development, division, and differentiated activity of the cell. Although general protein and enzyme syntheses have been studied as a function of limited phases of the cell cycle, very little information is available on glycoprotein, glycolipid, or lipid synthesis. This paper deals with the synthesis of protein, lipid, glycoprotein and glycolipids in the cell cycle of L5178Y cells.

MATERIALS AND METHODS

Cell Culture

L5178Y cells (mouse lymphoma cell line) were grown in suspension culture in sealed containers in Fischer's medium (14) with 10% horse serum and were utilized in the exponential growth phase. Fischer's medium in liquid form and horse serum were supplied by Grand Island Biological Co., Grand Island, New York. To the supplied medium, penicillin to 500 units/ml and streptomycin to 0.05 mg/ml

were added. Cell numbers were determined in a Coulter counter or by counting in a hemacytometer counting chamber.

Synchronization of Cells

Cells were synchronized by the method of Doida and Okada (12), by applying one treatment with excess thymidine followed by one treatment with Colcemid and deoxycytidine, except that in most experiments two to three times the stated volume of cell suspension ($1.5\text{--}3.0 \times 10^6$ cells) was used for each synchronization, and all volumes and concentrations of synchronizing agents were adjusted accordingly. For each experiment, 66–72 separate centrifuge bottles of cell suspension were synchronized in separate containers. In each instance, three synchronized centrifuge bottles were used for each of the 20 30-min labeling periods and six centrifuge bottles for zero controls. In some experiments, three to six synchronized centrifuge bottles were labeled for the entire 10 hr period, and in others additional synchronized volumes of cells were labeled at specific times for hydrolysis and identification of synthesized products.

Radioactively Labeled Compounds

The radioactively labeled compounds used and their specific activities were as follows: L-leucine-4,5- ^3H (50 Ci/mole), D-glucosamine-1- ^{14}C hydrochloride (50 Ci/mole), L-fucose- ^3H (UL) (10 Ci/mole), thymidine-methyl- ^3H (15 Ci/mole), and cholinemethyl- ^{14}C chloride (10 Ci/mole). For measurements of glycoprotein and glycolipid transferase activity with endogenous acceptors the following radioactive compounds were used: GDP-mannose- ^{14}C (120 Ci/mole), UDP-arabinose- ^{14}C (200 Ci/mole), UDP-galactose- ^{14}C (200 Ci/mole), UDP-glucose- ^{14}C (200 Ci/mole), and UDP-xylose- ^{14}C (200 Ci/mole). UDP-N-acetylgalactosamine- ^{14}C (200 Ci/mole) (16) and GDP-fucose- ^{14}C (110 Ci/mole) (8) were prepared as previously described. All initial compounds were purchased from New England Nuclear Corp. (Boston, Mass.), and the purity of each radioactive product was evaluated by paper chromatography or electrophoresis in several standard systems. In all cases no contaminating material was found.

Incorporation of Radioactive Precursors

The incorporation of radioactive precursors was measured by exposing the synchronized cells to a pulse label with the desired precursors for 30-min periods during 10 hr of the cell cycle (L5178Y generation time approximately 9.5 hr). For each experiment, 66 or more separate cell centrifuge bottles were simultaneously synchronized. Immediately after release from the Colcemid block, three synchronized volumes were centrifuged out of their medium, and

each was resuspended in 1 ml of Fischer's medium containing the desired radioactive precursors for 30 min in a 50 ml centrifuge tube at 37°C with gentle shaking in a Dubnoff (Precision Scientific Co., Chicago) metabolic shaker. The remaining synchronized cell cultures were allowed to continue through the cell cycle until the appropriate 30 min period when they were centrifuged and incubated as above. In this manner, 20 30-min periods were analyzed. All experiments were done as double labeled experiments, and the 1 ml of incubation medium contained, in different combinations of the tritium- and ¹⁴C-labeled compounds above, 1.25 μCi of the desired ¹⁴C-labeled compound and 12.5 μCi of the desired tritium-labeled compound. Immediately after the 30 min incubation the reaction was stopped by sedimenting the cells, and each of the respective tubes was immediately analyzed for "total incorporation," "trichloroacetic acid-insoluble," or "chloroform-soluble" material as given below. For each experiment zero controls were performed in six tubes by adding the 1 ml of medium containing the radioactively labeled compounds to the cells, immediately placing the tubes in ice, and performing the assays as given below (i.e., two tubes for total incorporation, two for secreted and cellular trichloroacetic acid-insoluble material, and two for secreted and cellular chloroform-soluble material). After counting the "zeros," these cps (usually less than 10% the incorporated levels) were subtracted from the appropriate incorporations for all results presented herein. It should be noted that, at each time period analyzed, the cells were synchronized in independent centrifuge tubes, and that for each of the three determinations at each time period (i.e., total, trichloroacetic acid-insoluble, and chloroform-soluble), independent synchronized cell populations were employed. At each time period incorporations were measured essentially by methods previously described (3, 8, 9).

Total Incorporation

At each 30 min interval for one of the three tubes incubated, total incorporation was measured in the following manner. After the 30 min incubation, the cells were immediately centrifuged at 3,000 g for 3 min, and the medium supernatant was removed by aspiration and was discarded. The cells in the pellet were washed two times with 2 ml of 0.9% NaCl; the cellular pellet was dissolved in 0.3 ml of 1 N NaOH and 0.15 ml of it was taken for a protein determination and 0.15 ml was plated on a glass fiber filter disc for counting in a PPO¹-POPOP²-toluene counting fluid in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). For each of the

¹ PPO = 2,5-Diphenyloxazole.

² POPOP = 1,4-Di-[2-(5-Phenyloxazolyl)] benzene.

radioactive precursors, this total incorporation fraction represents material incorporated into macromolecules, metabolized by the cell, or retained by membranes or in particulate matter in the cell.

Trichloroacetic Acid-Insoluble Fractions

At each 30 min interval for one of the three tubes incubated trichloroacetic acid-insoluble incorporation into both cellular and secreted material was measured in the following manner. After the 30 min incubation the cells were immediately centrifuged at 3,000 g for 3 min, and the medium supernatant was carefully removed with a Pasteur pipette and was separated from the cellular pellet. The supernatant fluid was precipitated with 1 ml of 30% trichloroacetic acid and was washed once with 10% trichloroacetic acid. The cellular pellet was suspended in 1% phosphotungstic acid in 0.5 N HCl and was washed once with 10% trichloroacetic acid. The resultant pellets were then extracted with ethanol:diethyl ether (2:1, v/v), and the supernatant was discarded. The pellet was dissolved in 0.3 ml of 1 N NaOH. Protein and radioactivity for these fractions were determined as above. The pellet for leucine radioactivity represents cellular protein, and the precipitate of the supernatant represents secreted protein. Glucosamine and fucose radioactivity in the precipitated supernatant is a measure of secreted glycoprotein, and in the cellular pellet it measures cellular glycoprotein. Thymidine radioactivity in the pellet measures cellular thymidine incorporation into DNA. In each extraction method, further washes did not lead to a change in specific activity.

Chloroform-Soluble Fractions

After each 30 min incubation, one of the three synchronized cell volumes was analyzed for chloroform-soluble radioactivity by the following procedure. After the incubation the cells were immediately centrifuged at 3,000 g for 3 min, and the medium supernatant was carefully removed with a Pasteur pipette from the cellular pellet. To the cellular pellet 1 ml of glass distilled water was added, and both the supernatant and the cellular pellet suspension were boiled immediately for 2 min. After boiling, both the cellular pellet and supernatant were brought to the temperature of melting ice, and 1 ml of chloroform:methanol (2:1 v/v) was added and the mixture was mixed vigorously for 0.5 min on a Vortex (Scientific Industries, Springfield, Mass.) mixer. The mixture was then centrifuged for 2 min at 1500 g, and the resultant aqueous portion plus the particulate layer at the water:chloroform interface was carefully removed and discarded. The chloroform extract was washed by adding 1 ml of water to the remaining chloroform and by repeating the mixing, centrifugation, and aspiration of the aqueous layer. The washed

chloroform extract was plated on glass fiber filter discs by repeated application, it was dried and counted in the liquid scintillation counter. As a control, samples were also analyzed in a gas flow counter (Beckman Instruments Inc.), which yielded identical results. Experiments were performed to test the reproducibility of the extraction procedure. Of 20 samples of L5178Y incorporating choline-¹⁴C and fucose-³H, each extracted by this method showed a range of values of less than 5% of the cpm. Tests performed on this material evaporated down were negative for protein (22) or amino acids by the ninhydrin reaction. For choline radioactivity, the chloroform extract of the cellular pellet represents cellular lipid; for glucosamine and fucose radioactivity, the chloroform extract represents cellular glycolipid (8). For the chloroform extract of the medium supernatant, choline radioactivity measures secreted or released lipid, and fucose and glucosamine radioactivity measures secreted glycolipid (8).

Measurement of Radioactive Substance Incorporated

In order to determine the identity of the incorporated radioactivity after the 30 min of incubation, the dried pellets and the residue of the chloroform-methanol extracts were hydrolyzed in acid and the hydrolyzate was chromatographed or subjected to electrophoresis against authentic standards. After chromatography or electrophoresis, the radioactivity was identified with a radioactivity strip counter. For choline-¹⁴C the hydrolysis conditions were 1 N HCl for 4 hr, for glucosamine-¹⁴C and fucose-³H 4 N HCl for 4 hr, and for leucine-³H 6 N HCl for 24 hr. After hydrolysis all samples were taken to dryness under vacuum over P₂O₅ and KOH. The samples were then made up to 50–100 μl with distilled water or 100% ethanol and spotted on paper for chromatography or electrophoresis as previously described (2, 9, 10). Fucose-³H, glucosamine-¹⁴C, and leucine-³H radioactivity was chromatographed in *n*-butanol-pyridine–0.1 N HCl (5:3:2, by vol), butanol-acetic acid–water (4:1:5, by vol) and isobutyric acid–ammonium hydroxide–water (57:4:39, by vol). Choline-¹⁴C radioactivity was subjected to paper electrophoresis in acetic acid/formic acid solution at pH 2 for 1 hr under constant voltage application.

Radioactive Assay Sample Size

All experiments were conducted as double labeled experiments in various combinations of the radioactive compounds; no corrections were made in the counting for quenching since all samples were handled in the same manner. Each of the radioactive compounds was analyzed the following number of times with a fresh harvest of synchronized cells: fucose-³H, 4; choline-¹⁴C, 4; leucine-³H, 5; glucosa-

mine-¹⁴C, 8; and thymidine-³H, 2. All data are presented as means ± SD except for thymidine, which is presented as means. Data for total incorporation are expressed per milligram of protein. Data for both cellular and secreted protein and glycoprotein are expressed per milligram of protein of the trichloroacetic acid-insoluble, ether: ethanol insoluble pellet of the cellular material. Because of the serum in the medium, the data on the secreted protein or glycoprotein could not be expressed per milligram of secreted protein. The data on cellular and secreted chloroform-soluble materials are expressed per average milligram of protein for the specific period under investigation, since it was impossible to perform direct protein analysis.

Protein

Protein was determined by the method of Lowry et al. (22). Crystalline bovine serum albumin was used as a standard.

Glycoprotein Transferases

Glycoprotein transferase activity with use of endogenous acceptors was assayed for as previously described (1, 7). A given culture of L5178Y cells (usually approximately 10⁸ cells) was homogenized in 5 ml of 0.1% Triton X-100 for 12 strokes with a Dounce (Blaessig Glass, Rochester, New York) homogenizer at 4°C and was extracted in the 0.1% Triton X-100 for 16 hr at 4°C with stirring. This extract was analyzed for glycoprotein transferase activity by incubating 10 μl of the desired nucleotide diphosphate monosaccharide-¹⁴C (approximately 40,000 cpm; 1.4 × 10⁻¹⁰ mole) with 50 μl of the extract at 37°C for 1 hr. After the 1 hr of incubation, the protein of the reaction mixture was precipitated with 1% phosphotungstic acid in 0.5 N HCl, was washed three times with 10% trichloroacetic acid, and washed once with ethanol:diethyl ether (2:1 v/v), was dissolved in 1 N NaOH, was plated on a glass fiber filter disc, and the radioactivity was determined in a liquid scintillation counter. All cells were in logarithmic phase.

Glycolipid Transferases

Glycolipid transferase activity with use of endogenous acceptors was assayed for as previously described (2, 3). After 1 hr of incubation with 0.1% Triton X-100 extracts prepared exactly as above for glycoprotein, 1 ml of distilled water was added to the reaction mixture, and the mixture was heated to 100°C for 2 min. After cooling in ice water, 1 ml of chloroform:methanol (2:1 v/v) was added, and the mixture was extracted by mixing in a Vortex mixer for 0.5 min. The extracts were centrifuged at 1500 g for 2 min, and the upper aqueous layer plus the chloroform:water interface was removed by aspira-

tion and discarded. The chloroform extract was washed twice with distilled water, plated on a glass fiber filter disc by repeated application, and counted in a liquid scintillation counter. All cells were in logarithmic phase before extraction.

RESULTS

Glycoprotein and Glycolipid Transferases

Initially it was deemed necessary to determine whether or not the L5178Y cells contained the necessary transferase enzymes and acceptors for incorporation of precursors into glycoprotein and glycolipid. The data in Table I indicate that Triton X-100 extracts of the L5178Y cells contained both transferase enzymes and acceptors for incorporation of monosaccharides into both glycoprotein and glycolipid. The data are the same whether expressed on a protein or cell basis. Extremely good endogenous glycoprotein synthesis occurred with GDP-fucose, GDP-mannose, UDP-glucose, UDP-galactose, and UDP-*N*-acetyl-galactosamine; there was little measurable difference in the amount of synthesis, although UDP-galactose gave the highest activity (2390 cpm/10⁷ cells). It should be noted that the results are not a measure of transferase activity, since either enzyme or acceptor, or both, can be limiting; however, the data do indicate that synthesis of glycoprotein with these sugars does occur. It should be noted that the results may be due to repair enzymes rather than enzymes actually involved in *in vivo* synthesis. Measurable, real synthesis of glycoprotein occurred with UDP-xylose and UDP-arabinose, but the activity was

extremely low (less than 100 cpm/10⁷ cells, in each instance). Hydrolysis of the reaction products and identification indicated that the fucose, glucose, galactose, and *N*-acetylgalactosamine were transferred unchanged; mannose was recovered as 50% mannose and 50% fucose.

Glycolipid synthesis also occurred in the Triton X-100 extracts of the L5178Y cells as shown in Table I. GDP-fucose, GDP-mannose, UDP-glucose, and UDP-galactose were all incorporated into endogenous lipid acceptors; the synthesis with GDP-mannose was by far the greatest (3360 cpm/10⁷ cells). Measurable but very low incorporation occurred with UDP-xylose, UDP-arabinose, and UDP-*N*-acetylgalactosamine. Hydrolysis of the reaction products and identification showed that fucose, mannose, glucose, galactose, and *N*-acetylgalactosamine were individually recovered 100% as the unchanged monosaccharide.

Protein Content of Cells

The total protein of the cell increased linearly after release from the mitotic block, and the amount of protein had doubled after about 9.5 hr of incubation (Fig. 1). The amount of protein was approximately 0.9 mg/10⁷ cells at 0 hr and 1.8 mg/10⁷ cells at 9.5 hr (Fig. 1). Measurements with the Coulter counter indicated a doubling at between 9.5 and 10 hr. L5178Y cells in logarithmic phase have approximately 1.2 mg of protein/10⁷ cells as determined by the described methods.

Identification of Material Transferred

Hydrolysis of the various incorporations after incubation with the various precursors and identi-

TABLE I
Glycoprotein and Glycolipid Transferases in L5178Y Cells with the Use of Endogenous Acceptors

Monosaccharide precursor	Glycoprotein		Glycolipid	
	cpm/10 ⁷ cells	cpm/mg protein	cpm/10 ⁷ cells	cpm/mg protein
GDP-fucose- ¹⁴ C	1,510 ± 100	680 ± 41	870 ± 61	384 ± 20
GDP-mannose- ¹⁴ C	1,960 ± 117	866 ± 36	3,360 ± 108	1,593 ± 72
UDP-glucose- ¹⁴ C	2,140 ± 111	947 ± 30	700 ± 62	310 ± 26
UDP-galactose- ¹⁴ C	2,390 ± 171	1,051 ± 87	760 ± 31	334 ± 30
UDP-xylose- ¹⁴ C	85 ± 4	40 ± 2	52 ± 1	24 ± 1
UDP-arabinose- ¹⁴ C	88 ± 6	41 ± 7	96 ± 9	45 ± 3
UDP- <i>N</i> -acetylgalactosamine- ¹⁴ C	1,450 ± 109	640 ± 36	130 ± 17	57 ± 6

Glycoprotein and glycolipid syntheses were measured as given in Methods. Background of the liquid scintillation counter (40 cpm) and 0 time controls (about 10% of assay cpm) were subtracted from each value. Means ± SD. Cells harvested and extracted in logarithmic growth phase.

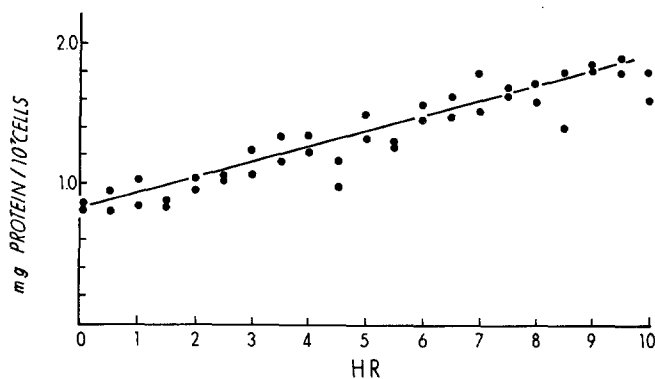


FIGURE 1 Protein content of cells after synchronization. Protein was determined after precipitation with 5% trichloroacetic acid, two washings with 5% trichloroacetic acid, and one washing with diethyl ether: ethanol (1:2, v/v). Data points are means of two determinations of two experiments.

TABLE II
Identification of Transferred Material

Material was incubated for a 30 min period, hydrolyzed, and assayed for as given in Methods. Data given as per cent incubated material recovered as indicated compound.

Compound incubated recovered as	Glucosamine			Fucose			Leucine Choline		
	Sialic acid	Galactosamine	Glucosamine	Glucose	Galactose	Mannose	Fucose	Leucine	Choline
Total incorporation	10	20	70	0	5	10	85	100	100
Secreted trichloroacetic acid-insoluble	50	0	50	0	0	0	100	100	—
Cellular trichloroacetic acid-insoluble	10	5	85	0	10	10	80	100	—
Secreted chloroform-soluble	9	8	83	0	0	20	80	—	100
Cellular chloroform-soluble	10	30	60	0	0	20	80	—	100

fication of the chemical nature of the radioactivity transferred resulted in the data presented in Table II. Both leucine and choline were recovered 100% as the unchanged compound in each of the three fractions measured. Thus in each instance leucine was incorporated directly into protein, and choline directly into lipid. Fucose, unexpectedly, underwent some transformation; in the cellular trichloroacetic acid-insoluble material the fucose was recovered as 80% fucose, 10% galactose, and 10% mannose. In the secreted trichloroacetic acid-insoluble material fucose was recovered 100% as fucose. In both the cellular and secreted chloroform-soluble materials the incubated fucose was recovered as 80% fucose and 20% mannose. Glucosamine radioactivity was recovered as 10% sialic acid, 5% galactosamine, and 85% glucosamine in the cellular trichloroacetic acid-insoluble material (Table II). In the secreted trichloroacetic acid-insoluble material glucosamine was recovered as 50% *N*-acetyl-neuraminic acid and 50% glucosamine, with no galactosamine present. In the cellular chloroform-soluble material the glucosa-

mine was recovered as 30% galactosamine, 10% sialic acid, and 60% glucosamine.

Total Incorporation

The data in Fig. 2 indicate that thymidine total incorporation by the L5178Y cells occurred primarily in the S stage of the cell cycle. Leucine total incorporation occurred throughout the cell cycle but was two to three times as high in the S period as in the G and M periods of the cycle. The total incorporation reached a plateau of between 300,000 and 330,000 cpm/mg protein in the S period (Fig. 2). Choline total incorporation was between 40,000 and 50,000 cpm/mg protein in the M and G₁ periods, increased to about double that level in the S period, and dropped to about 75,000 cpm/mg protein in the G₂ period. Total incorporations of fucose and glucosamine were similar, with incorporation of the sugars in the S period being about twice the level of the M, G₁, and G₂ periods.

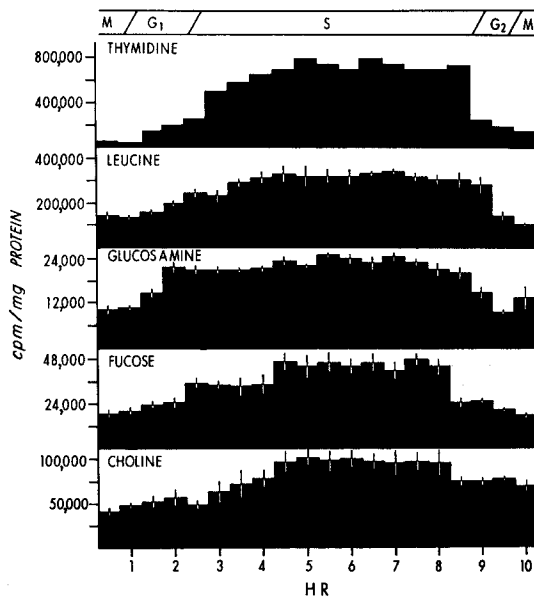


FIGURE 2 Total incorporation. Experiments began at 0 hr when the cells were released from the mitotic block and were pulse labeled for 0.5 hr intervals in discrete synchronized populations as given in Methods. Means \pm SD.

Cellular Trichloroacetic Acid-Insoluble Incorporation

The incorporation of thymidine into DNA occurred as expected in the S period and was greatly depressed in the M, G₁, and G₂ periods (Fig. 3). The maximum incorporation of thymidine into DNA occurred in the time period ending at 7.5 hr. Leucine incorporation into cellular protein occurred at approximately the same relative rates as DNA synthesis, with the highest synthesis of cellular protein occurring in the S period. The maximum synthesis of cellular protein occurred at the period ending at 7 hr. Synthesis of cellular glycoprotein as measured by glucosamine incorporation into trichloroacetic acid ethanol-diethyl ether insoluble material occurred also primarily during the S period, but the relative difference between the S and M periods in the glycoprotein synthesis was much more pronounced than for protein synthesis. The highest incorporation of glucosamine into glycoprotein occurred in the period ending at 5 hr. Glycoprotein synthesis as measured by fucose incorporation into glycoprotein (Fig. 3) paralleled the results found with glucosamine. Synthesis of fucose-containing glycoprotein

was highest during the S period and was greatly depressed during the M period (Fig. 3). The highest incorporation of fucose into glycoprotein occurred in the period ending at 5 hr.

Secreted Trichloroacetic Acid-Insoluble Incorporation

The results measuring the secretion of glycoprotein and protein are given in Fig. 4; the results from experiment to experiment were extremely variable as evidenced by the large standard deviations shown in the figure. Secretion of leucine-containing protein occurred throughout the cell cycle, with no generalizations being apparent; however, secretion was highest in late S and G₂ (Fig. 4). The highest secretion of leucine-containing protein was in the period ending at 8 hr. Secretion of glucosamine- and sialic acid-containing glycoprotein also occurred, in general, in late S and G₂. The highest secretion of glucosamine-containing glycoprotein was in the period ending at 9.5 hr. Secretion of fucose-containing glycoprotein was more constant throughout the cycle, with several peaks;

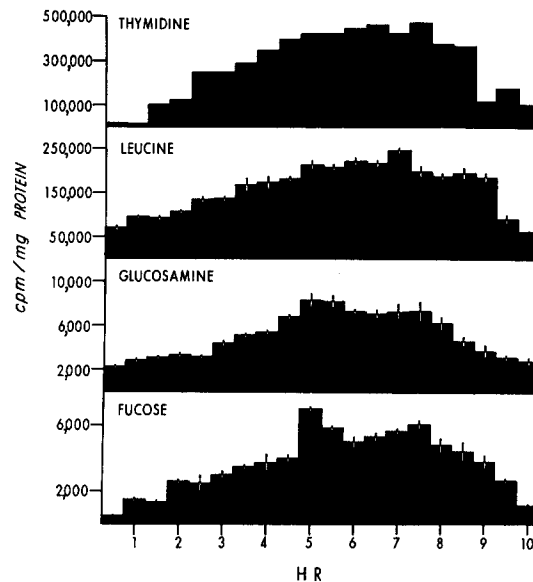


FIGURE 3 Cellular trichloroacetic acid-insoluble incorporation. Experiments began at 0 hr when the cells were released from the mitotic block and pulse labeled for 0.5 hr intervals in discrete synchronized populations as given in Methods. Means \pm SD. Choline-¹⁴C was not found in this fraction because it had previously been extracted with lipid solvents.

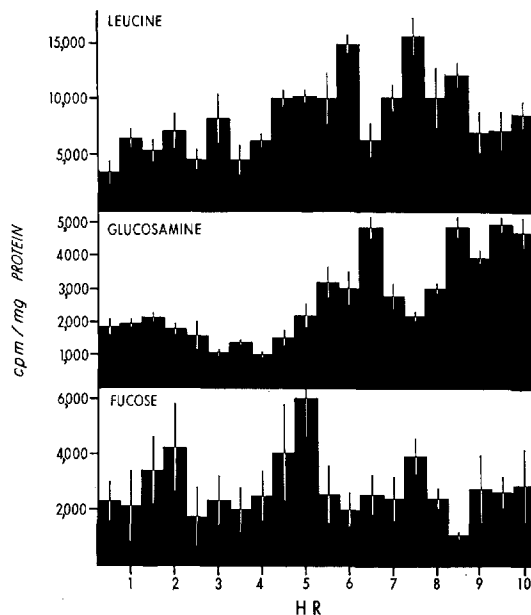


FIGURE 4 Secreted trichloroacetic acid-insoluble incorporation. Experiments began at 0 hr when the cells were released from the mitotic block and pulse labeled for 0.5 hr intervals in discrete synchronized populations as given in Methods. Means \pm sd. There was no net incorporation of choline- 14 C or thymidine- 3 H in this fraction.

the highest incorporation occurred in the period ending at 5 hr.

Cellular Chloroform-Soluble Incorporation

The results illustrating synthesis of lipid and glycolipid (Fig. 5) are the most dramatic and unexpected. The synthesis of choline-containing cellular lipid occurred in G_2 and M; there was no synthesis in G_1 or S. The highest incorporation of choline into lipid occurred in the period ending at 9 hr (Fig. 5). The synthesis of glucosamine-containing glycolipid also occurred only in the G_2 and M periods, with no incorporation in G_1 or S; the highest incorporation occurred in the period ending at 9.5 hr. The measurement of fucose-containing glycolipid was exactly similar to that of glucosamine; synthesis occurred in only G_2 and M. The highest incorporation of fucose into glycolipid was found in the period ending at 9.5 hr (Fig. 5).

Secreted Chloroform-Soluble Incorporation

The results in Fig. 6 indicate that both glycolipid and lipid were secreted exclusively in the first and

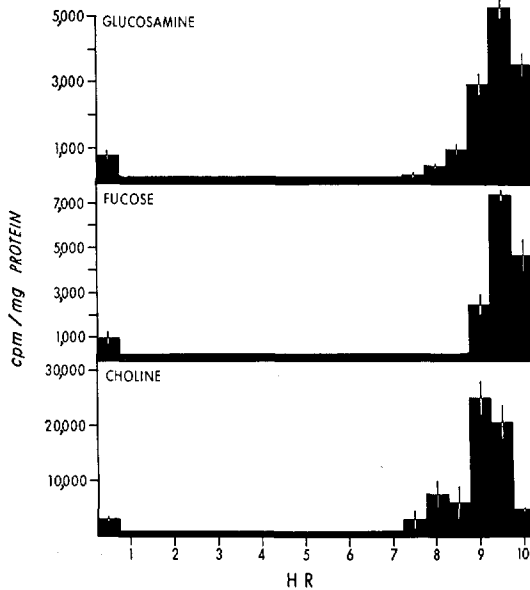


FIGURE 5 Cellular chloroform-soluble incorporation. Experiments began at 0 hr when the cells were released from the mitotic block and pulse labeled for 0.5 hr intervals in discrete synchronized populations as given in Methods. Means \pm sd. There was no net incorporation of leucine- 3 H or thymidine- 3 H in this fraction.

last 3½ hr of the experiments. The highest incorporation of choline into secreted lipid occurred in the period ending at 9 hr. The highest incorporation of both fucose and glucosamine into glycolipid occurred in the period ending at 10 hr.

Per Cent Incorporation of Sugars

Although quite variable (Figs. 3 and 4), the per cent of glucosamine- 14 C incorporated into secreted glycoprotein was 12%, while that incorporated into cellular glycoprotein was 88%; similarly, for fucose- 3 H the figures were 10 and 90%, respectively. The incorporation of glucosamine- 14 C into secreted lipid was 75%, and that incorporated into cellular glycolipid was 25%; the respective values for fucose- 3 H were 80 and 20%.

DISCUSSION

Although the division of the cell cycle into discrete stages is an arbitrary and simplified mechanism to describe the extremely complex series of events occurring in a continuum within the cell, this study and many others indicate that in fact most events in the cell cycle do occur at discrete times relative one to another. The results presented herein indi-

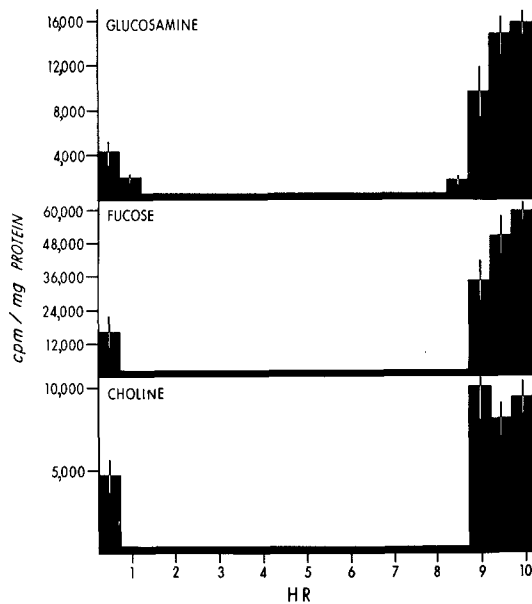


FIGURE 6 Secreted chloroform-soluble incorporation. Experiments began at 0 hr when the cells were released from the mitotic block and pulse labeled for 0.5 hr intervals in discrete synchronized populations as given in Methods. Means \pm SD. There was no net incorporation of leucine- 3 H or thymidine- 3 H in this fraction.

cate that in L5178Y cells protein is synthesized throughout the cell cycle, with the highest rates of synthesis occurring in the S and G₂ periods. Protein is also secreted throughout the cell cycle in the L5178Y cells, in agreement with the hypothesis that dividing cells do carry out differentiated activity, and in agreement with the results of Davies et al. (11) who found collagen synthesis to occur throughout the cell cycle in partially synchronized 3T6 aneuploid mouse cells. The synthesis of glycoprotein also occurred throughout the cell cycle, with the highest rates of synthesis occurring in S and G₂. Both protein and glycoprotein synthesis were depressed in the M and G₁ periods.

Secretion of glycoprotein occurred throughout the cell cycle; it is likely that the amino acid, leucine, and the sugars, glucosamine and fucose, were incorporated into the same glycoproteins (8), since most extracellular secreted proteins occur as glycoproteins (13). It is unlikely that the extracellular or secreted material studied herein is the result of cell lysis, since the chemical nature of the material was quite different in the cellular and secreted glycoprotein. This was especially true in

the case of the glucosamine-secreted glycoprotein in which the recovered radioactivity in the medium was 50% glucosamine and 50% sialic acid. The presence of sialic acid in the secreted material implies that this material might be surface membrane material ejected into the medium as suggested by Warren and Glick (32) in their studies on L cells.

The data on the synthesis of lipid and glycolipid, if accepted as a true indication of the sequence of events occurring in a normal nontraumatized cell, lead to some interesting speculations. The synthesis of glycolipid and lipid exclusively in the G₂ and M period may indicate the dependence of the synthesis on enzymes synthesized in the S period when protein synthesis is highest. The synthesis of lipid and glycolipid late in the cell cycle might signify that lipid and glycolipid macromolecules are the last molecules to be incorporated into membranous structures. The synthesis of lipid and glycolipid membrane precursors in only the G₂ and M periods may reflect the increased demand for lipid moieties for creation of new plasma membrane, if plasma membrane synthesis occurs at this time. Finally, the synthesis of glycolipid in only limited portions of the cell cycle, whereas glycoproteins are synthesized throughout the cell cycle, may explain why, when glucosamine- 14 C was incubated with nonsynchronized HeLa cells, much more radioactivity was found in glycoprotein than glycolipid (4).

In studies with HeLa cells (8, 16), it has been indicated that the biosynthesis of membrane glycoproteins occurs at the smooth membrane level of the cell after polypeptide chains are synthesized and released from the microsomes. It has been proposed (8) that, after being synthesized, the glycoproteins are integrated with lipid and other membrane components at the smooth membrane level to form membrane subunits. The data in this paper are compatible with the above hypotheses and indicate that synthesis of glycoprotein at the smooth membrane level occurs at its highest rate during the S period, and that the requirement of completed polypeptide acceptors may be responsible for this high rate of synthesis, since protein synthesis also occurs at its highest rate during the S period. The present data indicate that membrane subunit formation occurs at the smooth membrane level, with the addition of lipid components occurring last.

Because of its relatively long S period, the L5178Y cell is not the best cell line for the absolute

delineation of the cell cycle period in which the synthesis of membrane precursors occurs. However, the availability of a reliable method of synchronization made this the cell line of choice for this study. Further studies must be made with other synchronized cell lines and cell lines in which clean membrane fractions can be obtained.

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- Note Added in Proof:* It is of interest that Daniels (34) has reported a large transient increase in lipid synthesis per unit cell mass around the time of cell division (G₂-M period) in synchronized *Bacillus megaterium* KM and *Escherichia coli*. Thus, it appears that in both bacteria and mammalian cells a large increase in lipid, glycolipid, and membrane synthesis occurs in the G₂-M periods.
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