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INCORPORATION OF FUCOSE INTO HeLa CELL PLASMA MEMBRANES DURING THE CELL CYCLE

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SUMMARY

The metabolism of HeLa cell plasma membranes during the cell cycle was studied by following the incorporation of radioactive precursor L-[^{3}H]fucose into plasma membranes of synchronized cells. Maximal incorporation of the radioactive precursor was observed in late S phase of the cell cycle. This discrete period of increased incorporation of precursor into the plasma membranes implies the existence of a distinct control mechanism which may relate cell surface phenomena to the cell cycle.

INTRODUCTION

The use of synchronized cells in culture permits the study of temporal relationships between various cellular metabolic events and may help to elucidate the control mechanisms governing these events. Of particular interest is the possible existence of regulatory processes controlling the synthesis of various classes of macromolecules and assembly of organelles. Discrete periods of synthesis of such macromolecules during the cell cycle would imply the existence of distinct regulatory mechanisms; in this respect DNA, histones[1], and certain glycolipids[2] have been shown to be synthesized at a discrete stage in the cell cycle of animal cells. Our interest is in the metabolism of HeLa cell plasma membranes; by similar arguments, a discrete period of incorporation of precursors into plasma membrane components would imply the existence of some control mechanisms concerned with the synthesis of membranes. The elucidation of such controlled synthesis of membranes is of general interest since it could provide insights into possible regulatory interplay between cell surface changes and cell division.

In experiments described below the incorporation of L-[^{3}H]fucose into whole cells and plasma membranes of synchronized HeLa cells was studied. The plasma membranes were purified by a simple and rapid technique developed in this laboratory[3]. This method yields plasma membrane material in the form of intact ghosts and does not require the use of cross-linking agents or fixatives. L-Fucose was chosen as a precursor because it it is incorporated directly into macromolecules by animal cells[4], without transformation into glycogen or amino acids[5,6]. Also, the *in vivo* incorporation of L-fucose into the surface membrane of animal cells has been demonstrated by electron microscope radioautography in rat villi columnar cells[7]

and by studies on the distribution of L-[^{3}H]fucose in various subcellular fractions of HeLa cells[3], where it was shown that most of the L-[^{3}H]fucose incorporated by HeLa cells is recovered in the plasma membranes. In studies on the metabolism of L-[^{3}H]fucose in plasma membranes of HeLa cells we show that L-[^{3}H]fucose is maximally incorporated during late S phase of the cell cycle.

MATERIALS AND METHODS

Culture conditions, synchronization and radioactive labelling

HeLa S_3 cells were grown in suspension culture in Eagle's minimal essential medium supplemented with 3.5 % fetal calf serum, 3.5 % calf serum, penicillin and streptomycin. The cells were synchronized by the double thymidine block method as adapted for suspension cultures by Pagoulatos and Darnell[8]. This method gives at least 90 % synchrony. After release from the second thymidine block the cells were washed with medium and resuspended at a concentration of $4 \cdot 10^5$ per ml. The synthesis of DNA was monitored by 15-min pulses with [^{14}C]thymidine (New England Nuclear, 54.3 mC/mM, 0.5 μC/ml of cell suspension) at 1-h intervals, and the rate of incorporation of L-[^{3}H]fucose into trichloracetic acid precipitable material was estimated by 1-h pulses with L-[^{3}H]fucose (New England Nuclear, 4.3 C/mM, 5 μC/ml of cell suspension) every hour.

Purification of plasma membranes

The plasma membranes were purified as described by Atkinson and Summers[3], a procedure which recovers plasma membranes as intact ghosts (Fig. 1). The

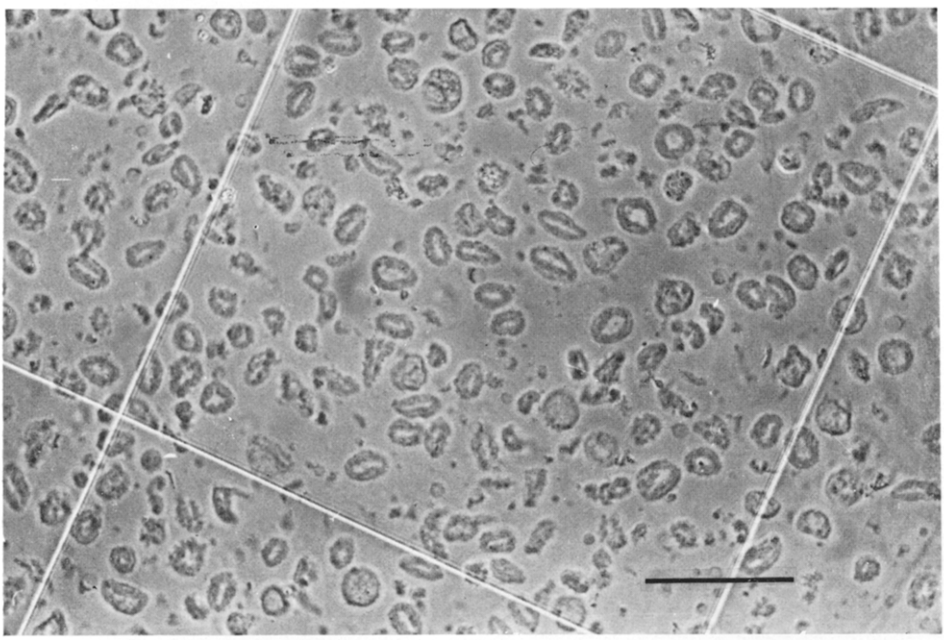

Fig. 1. Phase contrast micrograph of plasma membrane ghosts in a hemocytometer. The ghosts were purified by one cycle of zonal centrifugation (see Materials and methods). The photograph was taken of material suspended in Tris buffer. The cross lines are those of the hemocytometer. Bar = 30 μm.

entire purification required only 45 min which allowed the sequence of pulse labelings and membrane isolations shown in Fig. 3. Samples of $3.7 \cdot 10^7$ synchronized and labeled cells were washed, suspended in 4.0 ml of hypotonic Tris–HCl buffer, pH 8 (Tris buffer), and allowed to swell for 5 min. The cells were then ruptured by 10–20 gentle strokes in a stainless steel Dounce homogenizer (clearance 0.002 inch). The ion concentration of the resulting homogenate was adjusted to 1 mM Mg^{2+}, and the homogenate was centrifuged at 1000 × *g* for 15 sec to remove nuclei. The supernatant was saved and the pellet resuspended in 1 ml of Tris buffer containing 1 mM Mg^{2+}, and the mixture recentrifuged at 1000 × *g* for 5 sec. The supernatant was again saved and the pellet washed once more in a similar fashion. Most of the plasma membranes obtained after Dounce homogenization was contained in these three supernatants. The supernatants were combined and layered onto two discontinuous sucrose gradients, each composed of 15 ml of 30 % (w/w) sucrose on 5 ml of 45 % (w/w) sucrose in Tris buffer. The gradients were centrifuged at 7000 × *g* for 20 min and the plasma membrane ghosts were recovered from the 30–45 % interface with a syringe and No. 14 gauge canula. The sucrose solution in which the plasma membranes were suspended was diluted approx. 1:4 with Tris buffer

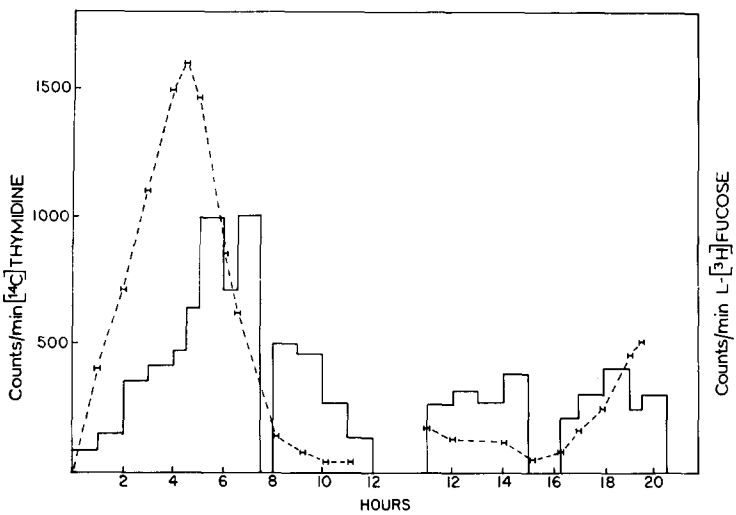

Fig. 2. Incorporation of [^{14}C]thymidine and L-[^{3}H]fucose by synchronized HeLa cells. A culture of 500 ml of synchronized HeLa cells was prepared in the following way: cells at $3 \cdot 10^5$ per ml were incubated for 15 h in the presence of 2 mM thymidine (thymidine, grade A, Calbiochem), washed with minimal essential medium supplemented with 3.5 % fetal calf serum and 3.5 % calf serum, resuspended in 500 ml of the same medium without thymidine for 10 h, and then 2 mM thymidine was added again for the next 15 h. At the end of this incubation the cells were washed with medium and resuspended at a concentration of $4 \cdot 10^5$ per ml. At indicated times 1-ml samples from the culture were pulse-labeled with 0.5 μC/ml of [^{14}C]thymidine (54.3 mC/mmole, New England Nuclear) for 15 min, or with 5 μC/ml of L-[^{3}H]fucose (4.3 C/mmole, New England Nuclear) for 1 h. All incubations were carried out at 37° with constant stirring. At the end of each pulse triplicate aliquots (0.2 ml) of the cell suspension were transferred into 3 ml of cold Earle's solution, centrifuged for 2 min at 1000 × *g*, resuspended in 1 ml of distilled water, and 1 ml of 10% trichloroacetic acid was added. Precipitates were collected on glass filters (Whatman, GFA), dried and 6 ml of scintillation fluid (2 vol. toluene, 1 vol. Triton X-100, Rohm and Haas) added. Radio activity was determined in a Beckman scintillation counter. – – – –, [^{14}C]thymidine, counts/min; ———, L-[^{3}H]fucose, counts/min.

and the ghosts pelleted by centrifugation at 7000 × *g* for 5 min. The pellet was resuspended in 1.0 ml of Tris buffer and the concentration of plasma membrane ghosts in a 1:10 dilution was determined by use of a hemocytometer (Fig. 1) and phase microscopy. The number of ghosts varied between 20 and 40 % of the initial number of cells used in the preparation. In this way their yield was estimated and the incorporation of L-[^{3}H]fucose normalized in the various preparations.

RESULTS

Maximum incorporation of [^{14}]thymidine into trichloroacetic acid precipitable material occurred at 4.5 h after release from the thymidine block. The peak of L-[^{3}H]fucose incorporation was observed about 2–3 h later, still within the S phase

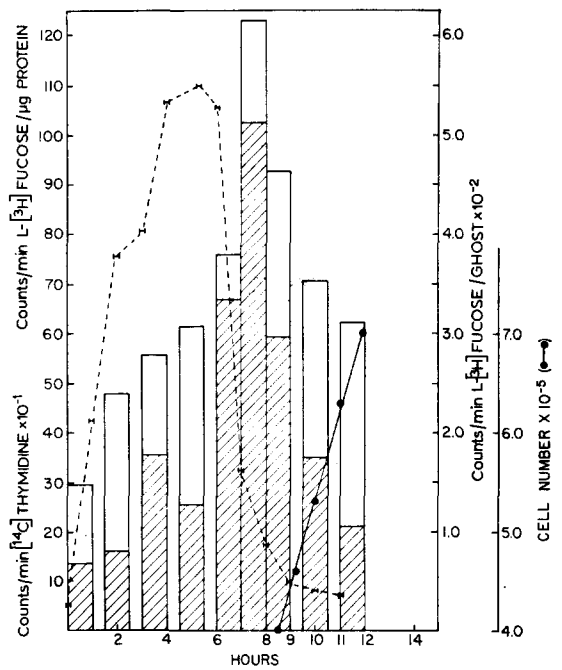

Fig. 3. Incorporation of L-[^{3}H]fucose into plasma membranes of HeLa cells at various times in the cell cycle. A culture of 1200 ml of synchronized cells at a concentration of $3.7 \cdot 10^5$ per ml was obtained with the use of double thymidine block (Fig. 2). At the indicated times after removal of the second thymidine block 100-ml samples of cells were removed from the stock culture, collected by centrifugation and resuspended in minimal essential medium (20 ml) at a concentration of $1.85 \cdot 10^6$ per ml; 0.01 ml of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid organic buffer, pH 7.4, was added to maintain a constant pH. The concentrated samples of cells were incubated for 1 h in the presence of L-[^{3}H]fucose (5 µC/ml), collected by centrifugation, washed twice with 40 ml of cold Earle's solution, and the plasma membranes prepared as described in Materials and methods. Duplicate 0.1-ml aliquots of the plasma membrane suspension were placed in 1 ml of distilled water, 0.1 ml of 10 % bovine serum albumin (0.25 mg/ml) carrier protein and 1.0 ml of 10 % trichloroacetic acid added. Precipitates were collected and radioactivity determined as before (Fig. 2). 0.3 ml of the plasma membrane suspension was used for protein determination by the method of Lowry *et al.*[9]. The radioactivity incorporated into the plasma membranes at indicated times in the cell cycle is expressed per ghost (striped bars) or per µg protein (blank bars). – – – –, incorporation of [^{14}C]thymidine into whole cells, determined as in Fig. 1; ●—●, number of cells per ml.

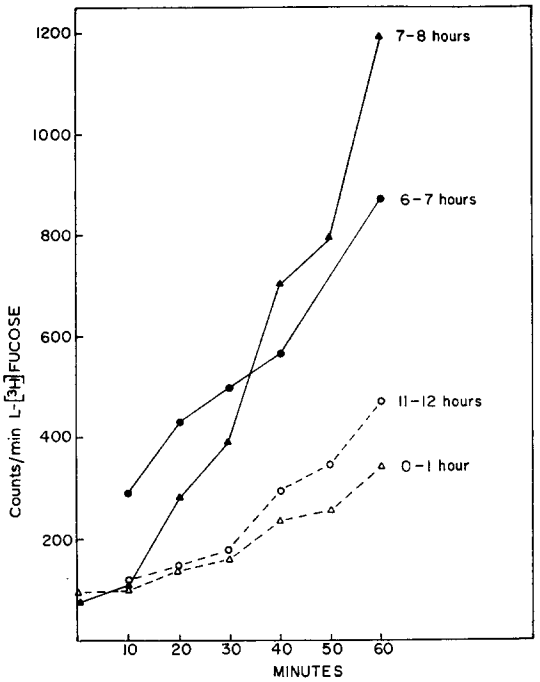

Fig. 4. Rate of incorporation of L-[^{3}H]fucose into trichloroacetic acid-insoluble material by synchronized HeLa cells during 1-h pulses at various times in the cell cycle. At 10-min intervals, duplicate 0.1-ml aliquots were withdrawn from the concentrated sample of cells used later for membrane preparation (Fig. 3), washed with cold Earle's solution and radioactivity determined as described in Fig. 2.

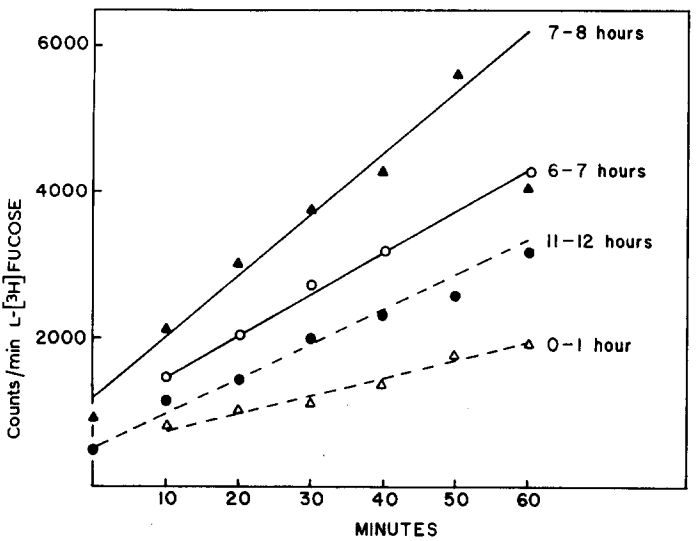

Fig. 5. Rates of uptake of L-[^{3}H]fucose into the soluble pool by synchronized cells at various times in the cell cycle. At 10-min intervals during the 1-h pulses with L-[^{3}H]fucose, duplicate 0.1-ml aliquots of the concentrated cell suspension (Fig. 2) were washed twice with cold Earle's solution, resuspended in 1.0 ml of distilled water and 0.1 ml of 10% sodium dodecyl sulfate was added. The resulting mixtures were transferred directly into scintillation vials to which 6 ml of scintillation fluid (Fig. 2) was added, and the radioactivity determined in a Beckman scintillation counter.

of the cell cycle. Later in the cell cycle, during G_1, the rate of incorporation of L-[^{3}H]fucose dropped again to near that observed in early S phase (Fig. 2). The increased rate of incorporation of L-[^{3}H]fucose by HeLa cells in late S phase reflects an increased incorporation of this precursor into the plasma membranes. This conclusion is based on experiments in which the incorporation of L-[^{3}H]fucose into the plasma membranes and into whole cell homogenates of pulse-labelled HeLa cells was compared at various times during S and G_2. When the radioactivity incorporated was compared and corrected for yield, approx. 50 % of the fucose label was found associated with plasma membranes at various times during the cell cycle. Similar results (Fig. 3) were obtained whether the incorporation of L-[^{3}H]fucose was expressed per ghost or per μg protein determined by the method of Lowry[9] in the membrane fraction.

At selected times during the cell cycle, the rates of uptake of L-[^{3}H]fucose into the trichloroacetic acid-soluble pool and into the trichloroacetic acid-insoluble material were determined by sampling the cultures at 10-min intervals during the L-[^{3}H]fucose pulses (Figs. 4 and 5). The rate of incorporation of L-[^{3}H]fucose into trichloroacetic acid-precipitable material at 7–8 h after removal of the second thymidine block (late S/early G_2) was 3.4 times that observed at 0–1 h after release from the block. By 11–12 h, when the cell number had almost doubled, the rate of incorporation had decreased to nearly that seen at 0–1 h. The rate of uptake of L-[^{3}H]fucose into the soluble pool also increased during the pulse at 7–8 h after release from the block, although it was only twice the rate of uptake during the 0–1 h pulse.

DISCUSSION

The results clearly indicate the existence of a regulatory mechanism operating during late S phase of the HeLa cell cycle. Among other possibilities, two explanations can be proposed for the changes observed: first, an increased rate of synthesis of plasma membrane glycoproteins during late S phase, and second, an increased specific activity of the GDP-fucose pool caused by changes in permeability properties transport or intermediate pathways. The data seem to indicate that both effects may take place, since we observe an increase in the uptake of L-[^{3}H]fucose into trichloroacetic acid-soluble pool and an even greater increase in the incorporation of this precursor into trichloroacetic acid-precipitable material. A direct determination of the specific activity of the GDP-fucose pool in synchronized cells at different times in the cell cycle could establish unequivocally the relation between the uptake of precursor and net synthesis of plasma membrane glycoproteins. Unfortunately, because the expected amounts of GDP-fucose per sample is of the order of only several picomoles, specific activity measurements based on the usual methods of spectrophotometry were not possible. However, alternative approaches to the measurements of the size and specific activity of the GDP-fucose pool in small numbers of cells are being explored.

Our results do not seem to confirm similar studies with KB cells[10] but are consistent with those of Bosmann and Winston[2], who found that most of glycoprotein synthesis in synchronized L5178Y cells, as indicated by the incorporation of L-fucose or glucosamine, takes place during S phase. Furthermore, the data presented here are supported by studies on changes in the amount of H blood group substance (a membrane component containing fucose) during the cell cycle of HeLa cells[11].

Our data are also consistent with some simple considerations of the requirements for increase in surface area of a cell during growth and division. During G_1, S, and G_2 cell volume doubles while the cell surface area increases by 60 %:

$$V_{\text{initial}} = 4/3\pi r^3 \qquad V_{\text{final}} = 2 \cdot 4/3\pi R^3$$

$$R/r = \sqrt[3]{2} = 1.26$$

$$S_{\text{final}}/S_{\text{initial}} = (1.26)^2 = 1.6$$

Here V_{initial} and V_{final} are the initial and final volumes of the growing cell, r and R are the respective radii, and S_{initial} and S_{final} refer to the initial and final surface areas of the growing cell.

At mitosis two daughter cells are formed whose total volume equals that of the parent cell. At the same time, however, total surface area of the two newly formed cells must increase by 25 % compared to the parent cell:

$$S_1 = 4\pi(1.26r)^2 \qquad S_2 = 2 \cdot 4\pi r^2$$

$$S_2/S_1 = 1.25$$

Here S_1 is the surface area of the parent cell; S_2, total surface area of the two daughter cells, and r is the radius. These estimates are minimal, since we assume the cells to be spherical. Comparing the relative duration of growth and division in the cell cycle, approx. 1/20 of the cycle is spent in mitosis whereas growth occurs continuously throughout the rest of the cycle. Thus, a sudden accumulation of the plasma membrane material just before the onset of cell division would be consistent with these requirements.

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