Equilibration of Fucosyl Glycoprotein Pools in HeLa Cells†

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ABSTRACT: The pool sizes, label equilibration times, and specific radioactivity relationships of fucosyl glycoproteins and precursors have been examined in exponentially growing HeLa S_3 cells (generation time about 23 h) using a quantitative radioisotopic approach. The specific radioactivity of the precursor GDP-fucose (pool size 0.52 ± 4% nmol/10^7 cells) equilibrates with radioactive fucose in the medium in about 1 h. 10^7 cells contain 5.3 ± 16% nmol of glycoprotein fucose of which 96–98% resides in or on the cell surfaces and is equilibrated isotopically within 22 h of labeling; 2% or less is in an internal pool, some of which is precursor to plasma membranes and some of which is released as soluble glycoprotein directly to the medium without random mixing with the plasma membrane glycoprotein. Because we cannot rule out the presence of internal free fucose, 2% of the total glycoprotein fucose could be in a degradative pathway being recycled internally before release of free fucose. In rate terms and in a particular culture where 10^7 cells contained 4.4 nmol of glycoprotein fucose, a total of 11.1 nmol of glycoprotein fucose is synthesized per generation (9–11% of the total cell glycoprotein fucose/h). Of this, 2.5 nmol of glycoprotein fucose per generation is released directly into the growth medium without mixing with the plasma membrane glycoprotein fucose. The small internal pool feeding glycoprotein fucose to the plasma membrane does so at the rate of 8.6 nmol/10^7 cells per generation, 4.2 nmol per generation of which, after mixing with the plasma membrane glycoprotein fucose, is ultimately released into the growth medium, 75–80% as free fucose. This release process is independent of cell density and the presence of serum in the growth medium.

Radioactive fucose, a glycoprotein precursor (Bekesi and Winzler 1967; Kraemer, 1971), labels almost exclusively glycoproteins in HeLa cells (Shen and Ginsburg, 1967; Atkinson, 1975), and is not randomized into other sugars (Kaufman and Ginsburg, 1968). Fucosylation of HeLa cell-surface glycoproteins is an intracellular event (Atkinson, 1975; Atkinson et al., 1976), a conclusion consistent with electron microscope observations made by Bennett and co-workers (1970, 1974) on the fucosylation of macromolecules in a variety of rat cells. However, relatively little is known either about the intracellular pools of fucosyl glycoprotein destined for the cell surface or about the fate of these molecules once they have reached or passed through the cell surface. Sequential relationships between intermediates in a pathway can be determined by comparing specific radioactivities (radioactivity/molar amount) at preequilibrium conditions. As [^{3}H]fucose-labeled precursor molecules move into a product pool, the fucose specific activity increases until equal to that of the precursor. Flow rates and pool sizes can be determined by quantitating the process. We believe that this approach, utilized by Kornfeld et al. (1965) to show that intracellular blood-group substance in hog gastric mucosa existed in several intracellular pools, is of general use to examine biosynthetic relationships between cellular membranes (and other) compartments.

Our previous paper (Yurchenco and Atkinson, 1975) reported a method by which the specific radioactivity of various fucosyl intermediates could be accurately determined in kinetic analyses. This paper reports rates and flow pathways of fucosyl molecules into and out of HeLa spinner cells, together with a mathematical description of the process.

Experimental Procedure

Cells, Radioactive Labeling, and Materials. HeLa S_3 cells were grown at 37 °C as previously described (Atkinson and Summers, 1971) or in Eagle's spinner medium (Eagle, 1959, except glucose was 2 g/L) without antibiotics. Cells tested monthly for mycoplasma contamination, were found to be free by both a culturing procedure (Levine et al., 1968) and by an enzymatic assay (Levine, 1972). At 37 °C, the cell-division time ranged from 22 to 24 h under these conditions and was determined by multiple-cell counting at several-hour intervals when required. Cells grew logarithmically in all experiments in the density range 1.5×10^5 cells/mL to 10×10^5 cells/mL. Labeled compounds used in this study were L-[1-^{14}C]fucose (48.66 mCi/mmol, New England Nuclear, Boston, Mass.), GDP[1]-L-[U-^{14}C]fucose (170 mCi/mmol, Amersham Searle, Arlington Heights, Ill.), [γ-^{32}P]ATP (initially >10 Ci/mmol, New England Nuclear), and L-[6-^{3}H]fucose (13.4 Ci/mmol, New England Nuclear). Cells were either labeled as they grew exponentially within their usual density range, or after concentration to $3–6 \times 10^6$ mL. Even at the latter densities the cells continued to grow exponentially for several hours. Cells were harvested after pulse labeling and washed for further processing as previously described (Atkinson, 1975).

Chromatography, Protein, and Radioactivity Determination. Paper and thin-layer chromatographic systems are the same as described in Yurchenco and Atkinson (1975) as were conditions for Sephadex chromatography. Protein and radioactivity determination are the same as described in Yurchenco

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[1] Abbreviations used are: GDP, guanosine diphosphate; ATP and ATPase, adenosine triphosphate and triphosphatase, respectively; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

and Atkinson (1975) and counting efficiencies were normalized as described.

Microassay of Fucose with Fucose Kinase, Specific Radioactivity. We have previously described (Yurchenco and Atkinson, 1975) a method for the quantitation of L-fucose with a sensitivity of 0.5 ± 0.05 nmol. GDP-fucose is extracted and purified from cells labeled with [^{3}H]fucose and then hydrolyzed to yield fucose which is further purified through Sephadex gel filtration and chromatographic steps (Yurchenco and Atkinson, 1975). The remaining insoluble cell material is hydrolyzed to release fucose from macromolecular material and is then purified from other contaminating sugars as described. L-Fucose kinase is used to transfer a terminal radioactive phosphate from [γ-^{32}P]ATP to [^{3}H]fucose according to the equation:

$$[^3\text{H}]\text{fucose} + [\gamma\text{-}^{32}\text{P}]\text{ATP} \xrightarrow{\text{fucose kinase}} [^3\text{H}]\text{fucose 1-}[^{32}\text{P}]\text{phosphate} + \text{ADP}$$

Since the specific activity of the [^{32}P]ATP is known, the quantity of fucose converted can be determined. From this, the specific radioactivity (cpm/mol) of [^{3}H]fucose-labeled GDP-fucose and glycoprotein fucose pools in the cells may be calculated. The recovery of [^{3}H]fucose was determined by the recovery of a known quantity of [^{14}C]fucose as previously described (Yurchenco and Atkinson, 1975). This, in conjunction with specific radioactivity measurement was used to determine the pool size. When absolute numbers were not required in equilibration rate experiments, relative specific radioactivity measurements were used by comparing the quantity of nonequilibrium (pulse labeled) [^{3}H]fucose radioactivity with equilibrium (long term labeled) [^{14}C]fucose radioactivity in GDP-fucose, total cell glycoprotein fucose, and plasma membrane glycoprotein fucose. Since relative specific radioactivity is a ratio, it is dimensionless. The term specific radioactivity is thus noted in the text as being either "cpm/nmol" or [^{3}H]/[^{14}C] ratio.

Isolation and Purification of HeLa Cell Plasma Membrane Ghosts. HeLa cell plasma membranes were prepared by one cycle of zonal centrifugation according to the procedure of Atkinson and Summers (1971) as described in detail by Atkinson (1973). When specifically noted, a subsequent isopycnic-banding purification step was used. Iodoacetate and azide were not used. Cells were pulse labeled, washed, and subsequently handled as previously described (Atkinson, 1975). For fucose determinations, the plasma membranes were dialyzed to get rid of excess sucrose and then hydrolyzed to release fucose in the same manner described for total cell glycoprotein fucose. Ouabain sensitive Na^+-K^+ dependent ATPase was enriched eight-fold in preparations made by one cycle of zonal centrifugation (E. Ceccarini, P. M. Novikoff, P. Atkinson, and A. B. Novikoff, manuscript in preparation) as was [^{3}H]fucosyl glycoprotein per mg of membrane protein. The enzyme was recovered to approximately the same extent as fucosyl glycoprotein (standardly 10–20% in these preparations) and the plasma membrane ghost structure. Plasma membranes were further purified by isopycnically banding them on a 20–50%)w/w) sucrose gradient (in 0.01 M Tris-HCl buffer, pH 8) using an SW27 rotor centrifuged 16 h at 25 000 rpm (Beckman L3-50 ultracentrifuge). Plasma membrane banded at a peak density of 1.16 g/mL; ouabain sensitive (Na^+,K^+)ATPase was enriched tenfold under these conditions (Ceccarini et al., manuscript in preparation).

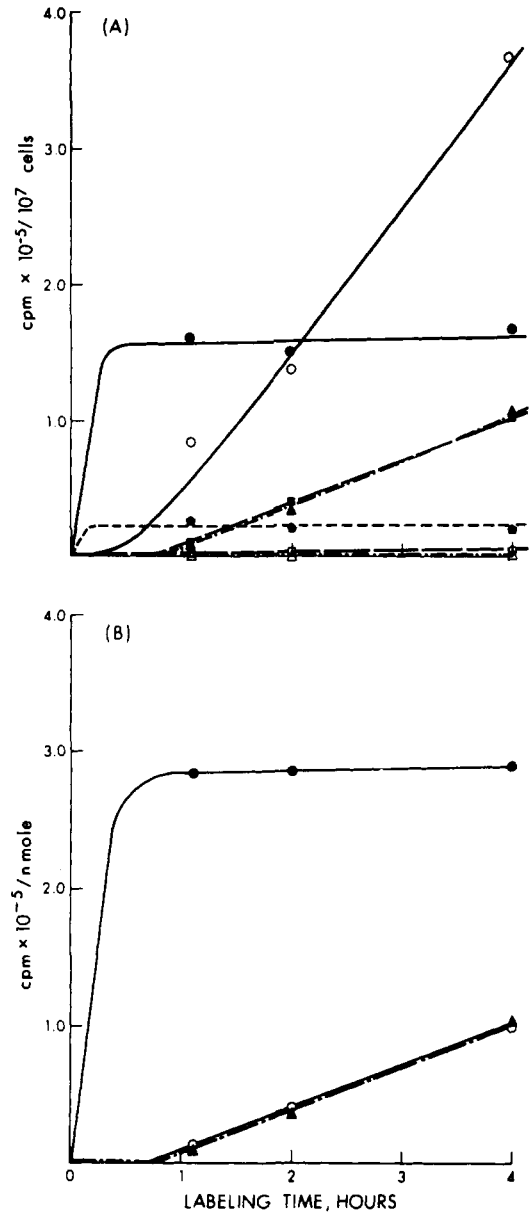

FIGURE 1: Pulse-label equilibration of L-[^{3}H]fucose in HeLa cells and growth medium. HeLa cells resuspended at a density of 3×10^6 cells/mL were labeled with 19 μCi/mL L-[^{3}H]fucose (1.67 μM fucose). After 1.08 h, 2 h, and 4 h, two aliquots of 2×10^8 cells were removed. One aliquot was used to prepare purified plasma membranes; the other was extracted with ethanol and processed to isolate GDP-fucose and total cell fucosyl glycoprotein. The fucose from all three fractions (GDP-fucose, total cell glycoprotein fucose, plasma membrane glycoprotein fucose) was released by hydrolysis and further purified by the steps of chromatography previously described, and a small aliquot from the ethanol extract was chromatographed on paper to separate GDP-fucose from fucose, fucose phosphate, and fuconic acid (Yurchenco and Atkinson, 1975). Four-milliliter aliquots of growth medium, after centrifugation at 33 000g for 45 min, were dialyzed for 3 days against three changes of 500 volumes of 0.85% NaCl. No low-molecular-weight labeled material remained, as determined by chromatography on Sephadex G-25. (A) This figure shows the incorporation of [^{3}H]fucose radioactivity (cpm/10^7 cells) as a function of the labeling time (hours). Total cell glycoprotein fucose (○), plasma-membrane glycoprotein fucose, not corrected for recovery (▲), growth medium macromolecular fucose (■), GDP-fucose (●), fucose (⬟), fucose 1-phosphate (□), fuconic acid (△). (B): Cells were labeled and fractionated as described for previous figure. The specific radioactivity (cpm/nmol of fucose) was determined for GDP-fucose, total cell glycoprotein fucose, and plasma-membrane glycoprotein fucose using the fucose kinase enzymatic-radiosotopic assay as described (Yurchenco and Atkinson, 1975). Specific radioactivity was plotted as a function of labeling time. GDP-fucose (●), total cell glycoprotein fucose (○), plasma membrane glycoprotein fucose (▲).

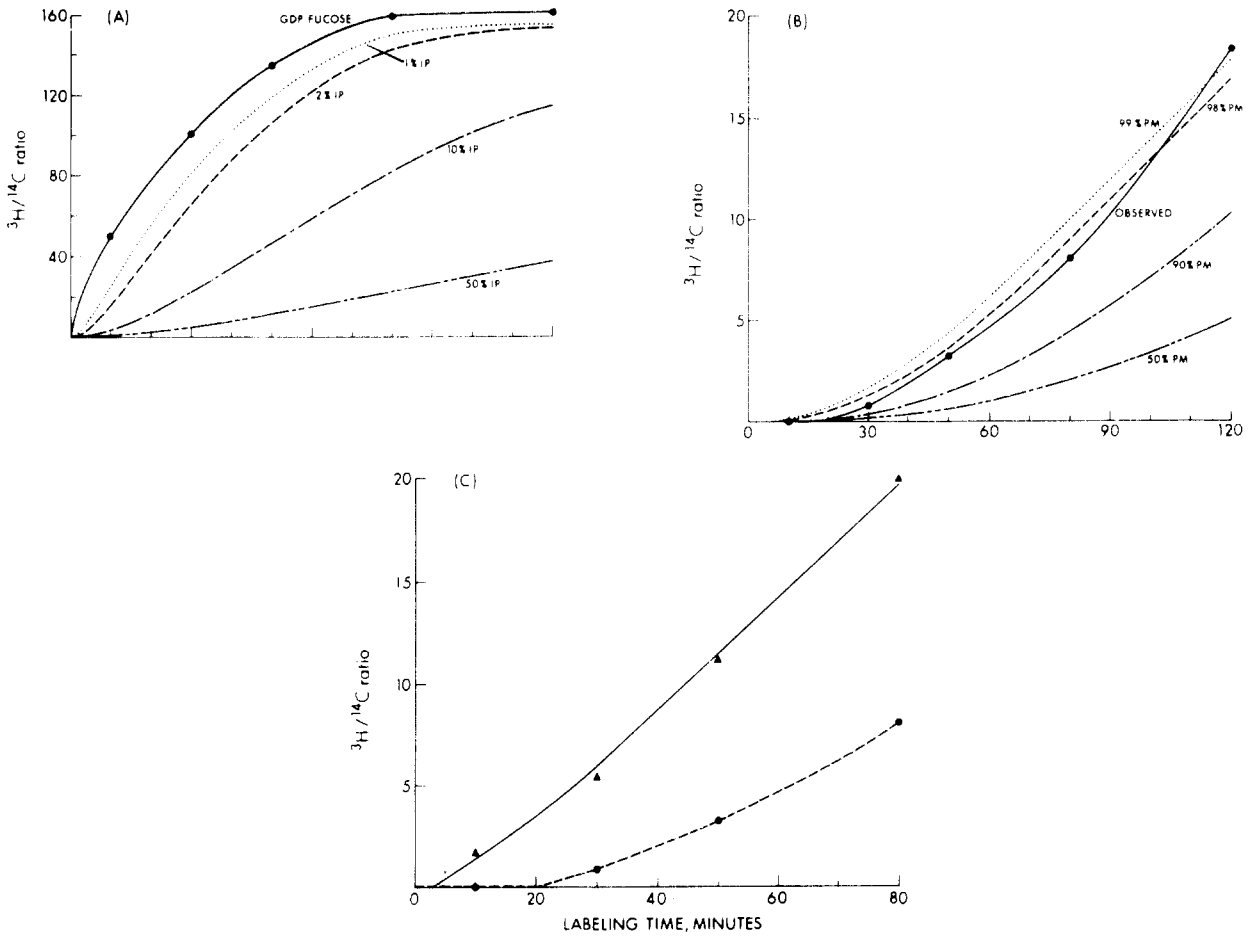

FIGURE 2: Predicted specific radioactivity $^3H/^{14}C$ of the internal pool after pulse labeling with [^{3}H]fucose. HeLa cells, labeled for 24 h with 0.03 μCi/mL of [^{14}C]fucose, were resuspended in fresh labeled medium of the same composition. Eighteen hours later, 635 mL of cells, at a density of 6.3×10^5/mL, were resuspended in 65 mL of the supernatant-labeled growth medium. After 25-min incubation at 37 °C, the cells were labeled with 25 μCi/mL of L-[^{3}H]fucose in a final volume of 66.7 mL (6×10^6 cells/mL). After 10, 30, 50, and 80 min, aliquots were removed (10^8 cells) and fractionated (Atkinson, 1973) on four discontinuous sucrose gradients into five bands. Band I (top of gradient), which was found to contain the GDP-fucose, was lyophilized and redissolved in 3 mL of water. 1.5 mL of this was chromatographed on Sephadex G-25 to determine the [^{3}H]fucose specific activity ($^3H/^{14}C$ ratio) of the nucleotide sugar. Band II (total 10 mL), which contains internal pool material, was diluted with Tris buffer, 1:0.6. 3.5 mL of this was layered into a 20–45% continuous sucrose gradient (35 mL) and centrifuged for 17 h at 25 000 rpm (4 °C) in an SW-27 rotor. Band IV, which contains the purified plasma membrane fraction, was not further purified. The 80–120-min portion of the GDP-fucose and plasma membrane specific activities of Figure 2A,B was obtained from Figure 1B where specific radioactivity was measured directly by the fucose kinase method. From the rate of increase of specific activity, plasma membrane/GDP-fucose specific activity ratios coincided to within 2% in the overlap region. This justifies the assumption that equilibration in this experiment occurred with the same kinetics as in Figure 1B. It follows $^3H/^{14}C$ ratio converts to ^{3}H cpm/nmol by multiplying by 1750. (A) The specific activities of internal glycoprotein fucose pools (IP) predicted from the measured values of the GDP-fucose at the various ^{3}H-equilibration times and of four theoretical sizes (0.044 nmol or 1% of total; 0.088 nmol or 2%, 0.44 nmol or 10%, 2.2 nmol or 50%) were predicted by numerical integration of eq 2 (described in the text) summing the label flowing into and out of the pool every 15 or 30 s after application of the label. GDP-fucose (●), 1% internal pool (• • •), 2% internal pool (– –), 10% internal pool (– – –), 50% internal pool (– – – –). (B) Using the data for internal pools generated in Figure 2B, and given that the plasma membrane pool size must be 4.4 nmol minus the IP size, numerical integration of eq 2 form (every 30–60 s after application of the label) was used to predict the plasma membrane specific activities. Observed plasma membrane equilibration (●), plasma membrane fed from a 1% internal fucosyl-glycoprotein pool (• • •), plasma membrane from a 2% internal pool (– –), plasma membrane from a 10% internal pool (– – –), plasma membrane from a 50% internal pool (– – – –). (C) Radioactivity was determined for Cl_3CCOOH precipitated peak fractions from gradient fraction II (▲) and gradient fraction IV (●).

Results

Flow Rates: Whole Cells, Plasma Membranes, and Medium. To determine rates of accumulation, exponentially growing cells (23-h generation time) were pulse labeled with [^{3}H]fucose for 1.08, 2, and 4 h. Intracellular fucose, fucose 1-phosphate, fuconic acid, and GDP-fucose were purified and radioactivity per cell number for these molecules was seen to plateau by 1 h of labeling (Figure 1A). Newly synthesized glycoprotein in the whole cells, in the purified plasma membranes, and in the growth medium, however, had not reached equilibrium with preexisting molecules during this labeling time and radioactivity accumulated with linearity from 1 to 4 h (Figure 1A). The quantity of newly synthesized material accumulating is not obtainable by this measurement alone and it is necessary to measure also the ratio of ^{3}H labeled to unlabeled molecules (specific radioactivity). Fucose from insoluble material (whole cell macromolecular material), purified plasma membranes, and GDP-fucose were assayed with fucose kinase and the specific ^{3}H radioactivities and pool sizes were determined. As may be suggested from the accumulation data (Figure 1A), GDP-[^{3}H]fucose specific radioactivity (also fucose, fucose 1-phosphate, fuconic acid) was observed to plateau by about 1 h, indicating equilibration of these pools with precursor radioactive fucose. Between 1 and 4 h, the whole cell macromolecular and plasma membrane [^{3}H]fucose specific radioactivities (cpm/nmol) were essentially identical and increased linearly (Figure 1B). Both extrapolated to the same

zero radioactivity time (~45 min). The average GDP-fucose specific radioactivity was 2.87×10^5 cpm/nmol of fucose and was essentially equilibrated by 1.08 h. Because GDP-fucose is the precursor to macromolecular fucose, the GDP-fucose specific radioactivity allows one to calculate the rate of flow of fucose into the macromolecular pools. From Figure 1A, the total rate of radioactivity accumulation into macromolecules (1.38×10^5 cpm per 10^7 cells per hour), divided by the GDP-fucose specific activity gives 11.1 nmol of total (cell + medium) macromolecular fucose synthesized per 10^7 cells per 23 h (generation). The rate of radioactivity accumulation in the cell alone (1.07×10^5 cpm h^{-1} $(10^7 \text{ cells})^{-1}$) divided by the GDP-fucose specific radioactivity gives 8.6 nmol of glycoprotein fucose synthesized per generation in 10^7 cells. Radioactivity accumulated in growth medium at the rate of 3.12×10^4 cpm h^{-1} $(10^7 \text{ cells})^{-1}$. This rate divided by the GDP-fucose specific radioactivity gives an accumulation rate of 2.5 nmol per generation of glycoprotein fucose in the medium. The specific radioactivity of fucose in the growth medium was calculated by summing the label and serum fucose (Yurchenco and Atkinson, 1975) and was determined to be 11.4 μCi/nmol (3.42×10^6 cpm/nmol). Therefore, there was a 1:12 dilution of specific radioactivity in converting exogenous [^{3}H]fucose to GDP-[^{3}H]fucose because the equilibrated GDP-fucose specific activity was 2.87×10^5 cpm/nmol, consistent with the result reported previously (Yurchenco and Atkinson, 1975).

Pool Sizes: Whole Cells and GDP-Fucose. Using the assay previously described (Yurchenco and Atkinson, 1975), the data of six determinations (two from the paper just cited) gave an average GDP-fucose pool size in 10^7 cells of 0.52 ± 0.05 (SD) nmol (0.47, 0.46, 0.57, 0.52, 0.57, 0.54 nmol) and an average whole cell glycoprotein fucose pool of 5.3 ± 1.9 (SD) nmol (6.1, 8.2, 6.2, 3.3, 3.6, 4.2 nmol). Fucose in total cell glycoprotein was 4.4 nmol/10^7 cells for the experiment described in Figure 2 and that recovered in the plasma membrane fraction in an average preparation was 0.91 nmol.

Internal Glycoprotein Fucose Pool Size. Since 2 h of labeling is a preequilibration labeling time for glycoprotein fucose (Figure 1B), it would have been expected that evidence of a precursor–product relationship in specific radioactivities would exist between a significantly sized internal pool and the plasma membrane fucosyl glycoprotein pool. An internal pool that approached the size of the plasma membrane fucose pool would, at 2 h of labeling, yield a plasma membrane with a much lower specific radioactivity as compared to the whole cell, while a small internal pool would yield a plasma membrane with nearly the same specific radioactivity. In fact, there was little measurable difference in specific radioactivity between cells and their plasma membranes (Figure 1B), suggesting that the internal fucosyl glycoprotein pool must be very small compared to the plasma membranes. To determine if more highly purifying the plasma membranes would affect these observations, membranes were prepared by one cycle of zonal centrifugation (as in Figure 1) and further purified by an isopycnic banding to their homogeneous density of 1.16 g/mL in a 20–50% (w/w) sucrose gradient. Though there was an increase in purification (as measured by [^{3}H]fucose cpm/μg of membrane protein) from 7.2 to 11.0 enrichment over the homogenate, there was little alteration in the specific radioactivity of whole cell and plasma membrane glycoprotein fucose (Table I). The difference in specific radioactivity of the isopycnically purified plasma membrane fucose and the whole cell macromolecular fucose is 13% (ratio of 0.87), which is probably a reflection of the size of the internal glycoprotein fucose pool precursor to plasma membranes. If the internal glycoprotein fucose pool specific radioactivity was known, its size could be directly calculated. However, since this pool cannot, at present, be fully isolated, other methods must be used to estimate its size. By calculating specific radioactivities for internal pools of various theoretical sizes using the measured value of the precursor GDP-fucose at any particular time, it is possible to predict the plasma membrane/whole cell specific activity ratio for a given internal pool size. With the aid of dilution formula,[2] a calculator was programmed to generate the expected glycoprotein fucose specific radioactivities by numerical integration during label equilibration in internal pools of varying sizes and also the plasma-membrane specific radioactivity which would arise from pools of these sizes (Figure 2A,B). These figures actually show equilibration rates based on relative specific radioactivity measurements; the absolute flow-rate numbers (for example, as in Figure 1B) are not important for this type of analysis. The observed plasma membrane/whole cell ratio of specific radioactivities at 120 min of labeling was 0.87 (for isopycnically banded membranes), consistent with an internal pool between 1 and 2% of the total (Table II). In the previous experiment (Figure 1B), the ratio was 0.88 for membranes prepared by one cycle of zonal centrifugation, also consistent with a 1–2% internal pool. At 65 min of labeling, the difference in specific radioactivities should be even more pronounced because of greater disequilibrium between precursor and product (Table II), especially for large internal pools. By the same type of analysis, ratios at 65-min labeling obtained from several experiments were consistent with internal pools 1% or less of the total (legend to Table II). We therefore have concluded that the precursor internal pool size is less than 2% of the total macromolecular fucose, and possibly 1% or less. Whether discontinuous sucrose gradient membranes or the more highly "purified" (with respect to cell protein; Table I) isopycnically banded membranes are used, the calculated internal pool sizes are approximately the same.

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[2] Consider a model in which labeled fucose flows through a series of glycoprotein and precursor pools (e.g., pools A and B) instantaneously diluted with unlabeled molecules in the first pool and flowing into the next where further dilution occurs. For constant pool sizes V_A and V_B (nmol) and a constant flow rate, u (nmoles/min) with label entering at constant specific radioactivity, C_i, the specific activity C_{At} of pool A (the first pool) at a given time t can be defined by

$$dC_{At} = (u/V_A)(C_i - C_{At})dt \quad (1)$$

and the specific activity of pool B (the second pool), C_{Bt}, is defined by

$$dC_{Bt} = (u/V_B)(C_{At} - C_{Bt})dt \quad (2)$$

The solution for the first pool is described by

$$C_{At} = C_i\,(1 - e^{-tu/V_A}) \quad (3)$$

The specific activity of the second pool, C_{Bt}, can most simply be determined by numerical integration of the known specific radioactivity of pool A at various times in which the flow of label into and out of the pool is summed over small (0.25–1 min) time intervals. It is more accurate, in the case of a growing cell, to consider a plasma membrane fucose pool (V_{Bt}) which increases in size, doubling in one generation time with the flow rate in (u_i) greater than the flow rate out (u_0). Hence, for the second pool,

$$dC_{Bt} = (1/V_{Bt})(u_iC_{At} - u_0C_{Bt})dt \quad (4)$$

where V_{Bt} increases linearly with time (V_{B_o} = pool size at $t = 0$) and

$$V_{Bt} = V_{B_o} + (u_i - u_0)t \quad (5)$$

Equation 5 describes an equilibration curve similar to the equation which assumes no pool expansion, and at 120-min labeling, comparing plasma membrane specific activities for the two equations, there was less than a 4% difference. Numerical integration was carried out using the no expansion equation (Figure 2A,B).

TABLE I: Plasma Membrane Purification.[a]

	[^{3}H] Fucose Sp Act. (cpm/nmol) $\times 10^{-4}$	Plasma Membrane/Cell Ratio	[^{3}H] Fucose (cpm/μg of Protein)	Plasma Membrane Cell Ratio
Whole cell glycoprotein fucose	5.72	1	45.3	1
Plasma membrane glycoprotein fucose (1 cycle centrifugation)	5.47	0.96	349	7.2
Plasma membrane glycoprotein fucose (isopycnic banding)	4.95	0.87	539	11.0

[a] Growing HeLa cells (6×10^8) were suspended at a concentration of 4×10^6/mL in fresh medium and labeled with 25 μCi/mL of L-[^{3}H]fucose for 2 h. The cells were cooled on ice, divided up into six aliquots, and washed three times with cold Earle's salt solution (pH 6.8). One of the aliquots was used to prepare the GDP-fucose and insoluble (macromolecular) fucose fractions. The remaining aliquots were used to prepare plasma membranes in a discontinuous sucrose gradient by zonal centrifugation and further purified by isopycnic banding at 1.16 g/mL. The fractions were then hydrolyzed to release fucose and purified as previously described. The fucose samples were then assayed with [γ-^{32}P]ATP and fucose kinase to determine their specific activity. It was determined that there were 0.54 nmol of GDP-fucose and 4.2 nmol of macromolecular fucose in 10^7 cells for this experiment; the GDP-fucose specific activity was 55.3×10^4 cpm/nmol.

TABLE II: Predicted Ratio of Plasma Membrane/Whole Cell Glycoprotein Fucose Specific Activities for Hypothetical Precursor Internal Pools.[a]

Internal Pool Size (nmol)	Precursor Internal Pool (% of Cell Total)	Plasma Membrane/Cell Ratio at 65-min Labeling	Plasma Membrane/Cell Ratio at 120 min Labeling
0.044	1[b]	0.80	0.92
0.088	2	0.65	0.84
0.44	10	0.22	0.47
2.20	50	0.09	0.23

[a] The above ratios were calculated from the data generated in Figure 2A,B and assume a 10-min lag (Atkinson, 1975) in movement of labeled fucose from the internal pool to plasma membrane. [b] Observed ratio of plasma membrane/cell specific radioactivities at 65 min of labeling in several experiments was 0.98, 0.95, 0.81, and 0.80, all consistent by the analysis in this table of an internal glycoprotein fucose pool of 1%.

The above results are based on a kinetic analysis but an estimate of the internal pool size can also be made from the known transit time of glycoprotein from an internal site of synthesis to the plasma membrane. The transit time of fucosyl glycoprotein from an internal membranous pool to the plasma membrane is ca. 10–15 min (Atkinson, 1975). This number was obtained by measuring the time difference in the appearance of fucosyl glycoprotein in the intact cells and the plasma membrane fraction purified from them. The internal glycoprotein fucose is located in a membranous component which banded symmetrically, though broadly, about a peak density of 1.13 g/mL in sucrose (Yurchenco, 1975; purified plasma membranes, by contrast, banded at 1.16 g/mL). The kinetics of equilibration of pulse labeled [^{3}H]glycoprotein fucose was observed in these two membranous fractions and [^{3}H]glycoprotein fucose accumulated in the 1.13 g/mL of glycoprotein earlier than 1.16 g/mL (plasma membrane) of glycoprotein with the same time difference (10–15 min; Figure 2C) as that previously published between the unfractionated cells and plasma membranes. The unwashed, isolated, plasma membrane ghosts (band IV; see legend to Figure 2) in the same experiment contained little (4.5%) detectable 10-min [^{3}H]-fucose pulse-labeled material. Therefore, it is possible to obtain plasma membranes with little measurable contamination with the internal 10-min pulse-labeled material. At the flow rates (see Figures 1A and 1B) we have established, 10 min of synthesis of fucosyl glycoprotein is 0.08 nmol/10^7 cells, approximately 2% of the total cell fucosyl glycoprotein. This estimate is an upper limit of the pool size and essentially confirms the conclusions we have drawn from numerical integration of the equilibration equations. We cannot, on the basis of the equilibration formulas alone, rule out that there is a large internal pool, say 50%, some or all of which contaminates plasma membranes during cell fractionation. However, it could not in the usual sense be precursor to plasma membrane because, for example, if 90% of an hypothesized 50% internal pool contaminated the plasma membranes the equilibration rates would resemble those for a small internal pool (Figure 2B). The only way to misinterpret the observed kinetics is for the possible large internal pool to be indistinguishable from plasma membrane in that no transit time exists between it and the plasma membrane; i.e., in cell fractionation, it would copurify with the plasma membranes. This clearly (Figure 2C) does not happen.

Equilibration of [^{3}H]Fucose in the Medium with Glycoprotein Fucose. When cells were grown in medium containing radioactive fucose, glycoprotein fucose was half equilibrated with newly synthesized glycoprotein in about 6 h and was wholly equilibrated by 24 h (Yurchenco, 1975). However, if growth medium was then renewed several times, the glycoprotein fucose increased again in specific radioactivity and reequilibrated (Figure 3). Each new increment in specific radioactivity after medium renewal was of lesser magnitude than the previous one and if a single best-fit curve is drawn, approximating more frequent growth medium changes, a $t_{1/2}$ label equilibration value of about 8.6 h is obtained. These observations would be consistent with the constant release of free fucose in the growth medium, thus reducing the specific radioactivity of the label, leading one to calculate a falsely high $t_{1/2}$ for equilibration.

Decay of [^{3}H]Fucosyl Glycoprotein Label from the Cell Surface When Prelabeled Cells Are Grown in Unlabeled Medium. The accumulation of free fucose in the medium from growing HeLa cells made it necessary to frequently renew the growth medium during a label-decay experiment to more correctly evaluate the half-life of cell surface fucosyl glycoprotein. Multiple medium renewal during the chase (Figure 4) had an effect of increasing the generation time to 30 h, although cells continued to divide logarithmically. Furthermore, about 5% dead cells was observed as compared to a value of 2% or less, which is usually observed. In the event of no cell-surface glycoprotein turnover, cells would produce only the new glycoprotein needed for cell doubling; i.e., unlabeled newly synthesized fucosyl glycoprotein would be mixed 1:1 with preexisting unlabeled glycoprotein after one generation time. Figure 4 shows the log plot of remaining glycoprotein fucose radioactivity per mL of medium expressed as a function of chase time (corrected for volume losses). Plotted in this form it is unnecessary to correct for dilution of label due to cell division. Loss of label from the cell surface was monophasic and loga-

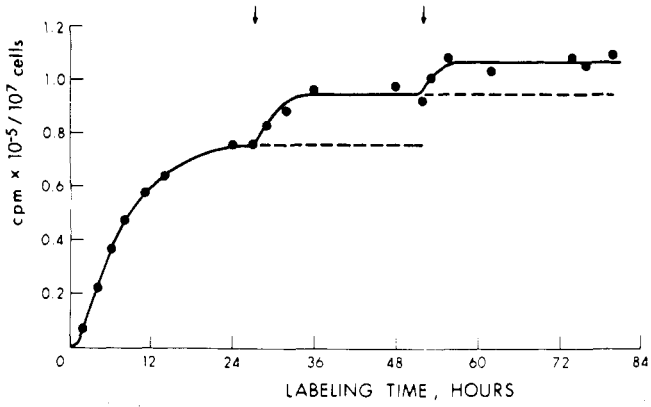

FIGURE 3: [^{3}H]fucose label equilibration of fucosyl glycoprotein pool when growth medium is periodically renewed. HeLa cells resuspended at a density of 4.1 × 10^5/mL were labeled with 1 μCi/mL of L-[^{3}H]fucose and, at the times indicated, 1-mL aliquots were removed. About every 24 h (indicated by arrows), the cells were pelleted by centrifugation and resuspended in fresh growth medium (from a single stock) labeled with L-[^{3}H]fucose. Trichloroacetic acid precipitable radioactivity per 10^7 cells is plotted as a function of labeling time. Dashed lines indicate the expected equilibration behavior if growth medium were not renewed.

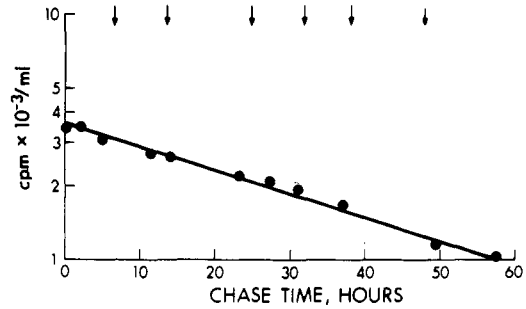

FIGURE 4: Loss of radioactivity from the cell surface of HeLa cells growing in label-free growth medium. HeLa cells were labeled to equilibrium for 4 days with 1 μCi/mL of L-[^{3}H]fucose (labeled growth medium was renewed daily), then resuspended in unlabeled growth medium at a density of 3.3 × 10^5/mL. Approximately every 8 h (arrows), the cells were resuspended in the same volume of fresh unlabeled medium. At the indicated times, multiple 1 mL aliquots were removed for determination of trichloroacetic precipitable radioactivity and cell counts. Semilogarithmic plot of radioactivity per mL of growth medium as a function of chase time is shown (corrected for volume losses). The generation time for cells in this experiment was 30 h.

rithmic. The half-life for the cell-surface glycoprotein label in these chase conditions was 30 h.

Characterization of [^{3}H]Fucose-Labeled Material Released From the Cell Surface into the Growth Medium. The label equilibration data (Figure 3) were consistent with the release of free fucose into the growth medium. To substantiate this, the material leaving labeled cells was characterized chromatographically (Figure 5). The loss of label from the cell was logarithmic; half of radioactivity per mL of cells in growth medium remained after 45.5 h. This half-life contrasted to the lower 30-h figure obtained when growth medium was changed every 8 h (Figure 4). The total cell and medium radioactivity remained constant (Figure 5). At various intervals from 6 to over 45 h of chase time, 70–80% of the labeled material appearing in the growth medium was in the form of free fucose (Figure 5) and the remainder, excluded with blue dextran from the Sephadex column, was macromolecular fucose. The included material in the monosaccharide region was further characterized and authenticated as fucose by paper chromatography. This release of free fucose seemed cell mediated rather than due to serum hydrolases, because, when the above experiment was repeated in serum-free growth medium after about 20-h chase, an average of 71% of the label released into growth medium chromatographed as free fucose. To determine the effect of cell density on the release of free fucose from the cell surface, the percentage of fucose accumulating in growth medium was measured at low and high cell densities at early chase times. After resuspension of cells at about 6 × 10^5/mL (low density) and 3 × 10^6/mL (high density) in unlabeled medium, cell and growth medium radioactivity were determined. At 4.25 h, chase time, low-density cells released 54% free fucose and 46% macromolecular fucose into the growth medium, and high-density cells released 62% free fucose and 38% glycoprotein fucose. The numbers are fairly close to each other, indicating that cell density has only small, if any, effect on the proportion of free and glycoprotein-bound fucose released from the HeLa-cell surface.

From 20 min to 6 h of chase time, the percentage of the total radioactivity released from the cell surface appearing as free fucose (determined by Sephadex G-25 and paper chromatography; see Materials and Methods) increased from 42 to over 70% and eventually plateauing at about 78%. The interpretation of this data is to be found in the Discussion.

Origin of Glycoprotein Fucose Appearing in Growth Medium. To determine the origin of gylcoprotein fucose appearing in growth medium, the specific radioactivity of growth medium glycoprotein fucose was compared with that of cell-surface glycoprotein fucose and GDP-fucose. Since the serum in growth medium contains fucosyl glycoprotein (Bekesi and Winzler, 1967), one would need to grow cells in serum-free medium in order to measure the specific activities of cell-derived glycoprotein fucose using the enzymatic-isotopic assay. This was circumvented by labeling cells to equilibrium with [^{14}C]fucose. (By 37 h of equilibrium labeling with [^{14}C]fucose, cpm/cell number remained constant.) Thus, relative specific radioactivity of a short-term [^{3}H]fucose label could be measured by ^{3}H/^{14}C ratios (as was also used in Figure 2) and, by this method, the specific activity of glycoprotein fucose accumulating in the growth medium was seen to be much higher than that of the plasma membrane[3] (Figure 6); in fact, the time-averaged specific radioactivity of glycoprotein released into the medium was nearly the same (70–85%) as the GDP-fucose pool. This would not be possible if all of the released glycoprotein were directly originating from the plasma membrane. Thus, the most direct origin (70–85%) of released glycoprotein is not the cell surface glycoprotein; neither can it mix to any significant extent with cell-surface glycoprotein. This conclusion is further supported by its apparent linear accumulation as from a small precursor pool, since derivation from a large nonequilibrated pool (i.e., plasma membrane) would result in a sigmoidal accumulation curve.

[3] The cell-pool specific activities cannot be compared directly to the accumulated relative specific radioactivity of the growth medium. Consider material released only from the cell surface at a given moment in time; this material will leave the cell surface at the cell-surface specific radioactivity; however, it will mix with all of the previously accumulated material which entered the growth medium at lower specific radioactivities; i.e., the measured specific activity of the medium represents the average of all accumulated released material. A time average of the GDP-fucose and cell surface glycoprotein fucose specific radioactivity curves (corrected for logarithmic increase in cell number) indicates that the expected values of material in the medium would be at any time if material entered the growth medium solely at the specific radioactivity of either "B" pool (see Figure 7) or cell-surface glycoprotein fucose.

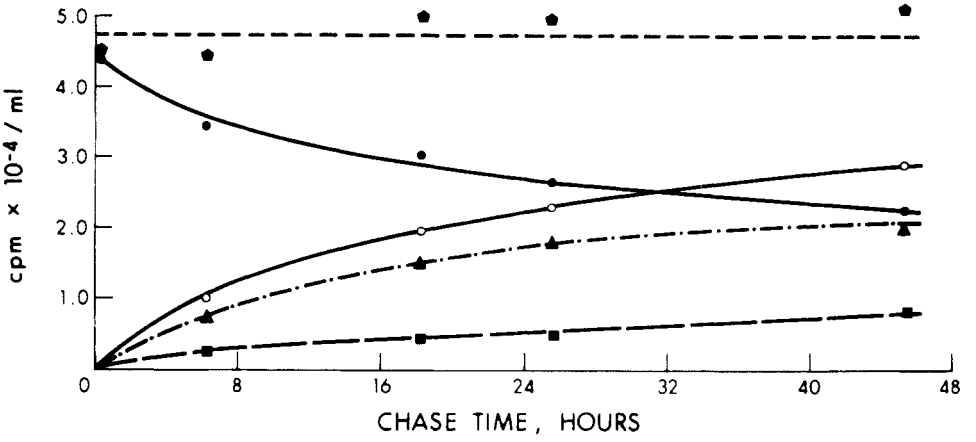

FIGURE 5: Release of free fucose from the HeLa cell surface. HeLa cells were resuspended in 200 mL of growth medium at a density of 2.6×10^5/mL labeled with 1 μCi/mL of L-[^{3}H]fucose for 26 h, rapidly washed three times with warm unlabeled growth medium, and finally suspended in 200 mL of fresh unlabeled growth medium. At the times indicated, several 1 mL aliquots were removed for determination of cell number, whole cell radioactivity and total growth medium radioactivity. One-milliliter aliquots of cell-free growth medium were chromatographed on Sephadex G-25 for determination of the amount of free fucose and glycoprotein fucose released from the cell. Labeled material migrating as free fucose was authenticated by paper chromatography (Yurchenco and Atkinson, 1975). When the above pulse chase experiment was repeated using serum-free growth medium, ~71% of the radioactivity after 20 h of chase chromatographed as free fucose. Total radioactivity per mL of growth medium (⬟); cell radioactivity per mL (●); total growth medium radioactivity per mL (○); medium fucose per mL (▲); medium glycoprotein fucose per mL (■). The generation time for cells in this experiment was 24 h.

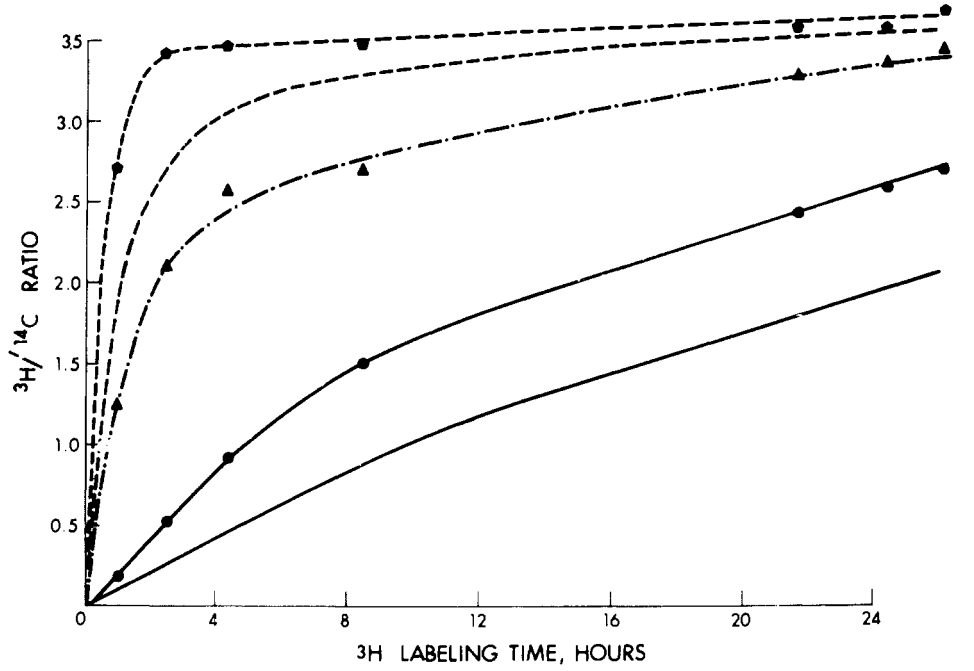

FIGURE 6: Relative specific radioactivity of cell-derived growth medium glycoprotein fucose at preequilibrium-labeling conditions. HeLa cells were suspended in 500 mL of growth medium and labeled with 0.1 μCi/mL of L-[^{14}C]fucose with labeled medium renewed after 24 h. After 37 h of labeling, the cells were resuspended in [^{14}C]fucose labeled growth medium (4.2×10^5/mL) and after 4 h, 1.23 μCi/mL of L-[^{3}H]fucose was added initiating the tritium pulse. At zero time and the times shown, 147-mL aliquots were removed (the growth medium supernatant was saved for dialysis) and the cells were extracted with ethanol. The GDP-fucose and fucose from glycoproteins were purified as previously described. Growth medium glycoprotein fucose specific activity ($^3H/^{14}C$ ratio) (▲), cell GDP-L-fucose specific activity (⬟), and cell glycoprotein fucose specific activity (●) are plotted as a function of L-[^{3}H]fucose labeling time. In order to compare cell fraction specific activities, the time averages of GDP-fucose specific activity (- - -) and cell glycoprotein fucose specific activity (—) were calculated (see Discussion).

Discussion

Short-Term Labeling of HeLa Cells. A lag of some 10–15 min between incorporation of radioactive fucose into total cell fucosyl glycoprotein and plasma membrane fucosyl glycoprotein has been shown (Atkinson, 1973; Yurchenco, 1975) to be due to the transit time between precursor internal glycoprotein and the cell-surface glycoprotein. Data from several experiments (Tables I and II), when compared to predicted ratios of plasma membrane/whole cell fucose specific activities arising from hypothetical internal pools of various sizes (Table II), were consistent with the conclusion that the size of the internal precursor pool is not more than 2% of the cell macromolecular fucose. The possibility of an internal glycoprotein fucose pool which has no outflow (i.e., *not* precursor to any other pools) but simply fluctuates in size each generation was not addressed in the text, but was considered unlikely for the following reasons. If HeLa cells are labeled with [^{3}H]fucose and then "chased" by diluting out label with 40 mM fucose (Atkinson, 1975), all of the label can be chased into the plasma

membrane.[4] (Though this is observed in uninfected HeLa, in vesicular stomatitis virus infected HeLa not all fucosyl-G protein could be chased from within the cell; Atkinson et al., 1976). In another study (Atkinson, 1973), cells were labeled to equilibrium with [^{14}C]fucose, pulsed with [^{3}H]fucose for 25 min, and then chased. Within 30 min from the start of the chase, the whole cell and plasma membrane $^{3}H/^{14}C$ specific radioactivity ratios were equal. Again, this could not occur if there existed an internal pool with no outflow. This conclusion may not be a general one for other cell types, since cultured rat hepatoma cells appeared to have an approximately 50% internal fucosyl glycoprotein pool (Tweto and Doyle, 1976); however, it is not known if this pool is precursor to any other. Also, a difference in cell types (e.g., secretory vs. mainly nonsecretory cells) may account for the differences in behavior. The problem of contamination of the isolated plasma membranes with internal membranous component containing fucosyl glycoprotein was addressed in the text: neither the equilibration kinetics (Figure 2A,B) nor the rate of appearance of pulse-labeled glycoprotein in the plasma membranes (Figure 2C) was consistent with a large internal pool extensively contaminating the plasma membrane fraction.

Devreotes and Fambrough (1975) observed that in developing muscle cells, cell-surface acetylcholine receptor sites labeled with [^{125}I]α-bungarotoxin may have a 10% internal precursor pool. After assembly, surface molecules are internalized and degraded, and the products (labeled tyrosine) enter the growth medium. It is possible HeLa cell-surface fucosyl glycoprotein is likewise internalized and degraded forming free fucose which then is released into the growth medium, since free fucose does represent 4% of the total cell-soluble fucose (Yurchenco and Atkinson, 1975).[5] If its origin is cell-surface glycoprotein fucose, it would have the same specific radioactivity, and it can be estimated that there could be a maximum of 0.3 nmol of internal free fucose at any instant. In addition, there would be 0.1 nmol of glycoprotein fucose, since 25% of cell surface derived fucose which eventually reaches the growth medium remains in macromolecular form. Because the outflow rate from cell-surface fucose was 0.18 nmol/h, this internalized material could accumulate up to about 2 h inside the cell before release into growth medium. Although our data are not inconsistent with an internal degradative mechanism, further investigation is required to substantiate its hypothesis. If correct, 2% of the total macromolecular fucose additional to the plasma membrane precursor pool would be within the cell (at the specific radioactivity of the plasma membrane fucose), leaving 96% associated with the cell surface.

The logarithmic loss of label in a chase experiment is monophasic (Figure 4), over the time periods measured (20 min to 58 h). This is interpreted as indicating that all of the major species of cell-surface glycoproteins exhibit the same rate of turnover; if a species of significant percentage of the total had a very different turnover rate, a biphasic decay pattern would be seen, for example, similar to that observed in lymphocytes (Melchers and Anderson, 1973).

Turnover Rates Estimated From Label-Equilibration Kinetics. A general mathematical discussion of membrane turnover has been given by Schimke (1975) and Cleaver (1967), and in this framework we define the turnover rate (T) of glycoprotein fucose as equal to that synthesized in excess of growth replacement (i.e., that lost from the cell excluding that which is released directly to the medium) in a cell generation divided by that present in the plasma membrane. Thus, for fucosyl glycoprotein in HeLa cells, $T = ((8.6 \text{ nmol}/23 \text{ h}) - 4.4 \text{ nmol})/4.4 \text{ nmol}$, which equals a turnover rate of 95% per generation. Turnover for GDP-fucose, similarly is 2120% per generation. At half-equilibration, $t_{1/2} = (V/u) \ln 2$ (derived from eq 3). From Figure 3 it can be determined that the halftime for equilibration is 8.6 h; the equation predicts 8.2 (or 9.8 h when the expanding pool equation is used (eq 4)). Another equation for turnover with fewer assumptions can be derived for pulse-chase experiments (label "washout"). In these conditions, if molecules are randomly mixed and lost then one would predict a logarithmic decay, as was the case in HeLa cells (Figure 4). Since the halftime for decay is expressed by $t_{1/2} = (\ln 2)/m$ (where m is the decay slope), $T = (g \ln 2)/t_{1/2}$ (g = generation time). In a chase experiment, turnover rates can be validly determined for cell-surface fucosyl glycoproteins only if precursor pools are small in size, rapidly lose their labeled molecules as compared to the cell surface, and if recycling of labeled [^{3}H]fucose back into the internal pools and thence to the cell surface is minimal. For the HeLa-cell system, the largest precursor pool is GDP-fucose; however, this pool rapidly deequilibrates, as shown above, because of its high turnover rate. If the growth medium is renewed frequently to remove accumulated free fucose, recycling of label will not occur significantly. Under these conditions, a $t_{1/2}$ of 30 h of chase time was measured (Figure 4; in this experiment $g \simeq 30$ h). Substituting into the above equation for $t_{1/2}$ gives a turnover rate of about 70% per generation. This value compares fairly well with a value directly calculated from the observed rate of accumulation of newly synthesized glycoprotein fucose of 95%. When growth medium was not renewed (Figure 5), a decay half-time ($t_{1/2}$) of 46 h was obtained, reflecting that released labeled fucose was being reutilized.

Fate of Cell-Surface Macromolecules. These turnover observations confirm those of Kaufman and Ginsburg (1968) who have previously shown that fucosyl glycoprotein decays with a half-time similar to the rate expected in a chase where the medium is not changed. Warren and Glick (1969) have investigated cell-surface turnover in L cells using labeled glucose, glucosamine, valine, and leucine, and concluded that in the growing cell the label is incorporated into purified plasma membranes with net increase in plasma membrane and little, if any, turnover. The present study shows that rapidly growing HeLa S_3 cells do turn over their membrane glycoproteins in agreement with data for L cells (Hubbard and Cohn, 1975), where different from HeLa, the turnover of iodinated proteins was found to be biphasic. HeLa S_3 cell-surface proteins labeled with ^{125}I by the lactoperoxidase method (Huang et al., 1973) had a $t_{1/2}$ for decay of about 21 h, and a turnover rate of 76% would be calculated given a 23-h generation time. This value falls into the range calculated by us for the turnover of fucosyl glycoprotein and, therefore, it appears that glycoproteins and those proteins which are iodinated turn over at approximately the same rate. These authors found that only 20% of the ^{125}I label released into the growth medium was macromolecular; the remaining 80% was acid soluble and, hence, due to degradation. Hartzell and Fambrough (1973) noted an internalization of cell-surface bound α-bungarotoxin labeled with ^{125}I in muscle cells as did Cook et al. (1976) for HeLa S_3 cell-surface ouabain binding sites. These (ATPase)

[4] Internal-pool fucose label was determined by subtracting plasma membrane radioactivity corrected for recovery from whole cell radioactivity (Atkinson, 1975).

[5] In Figure 1A, 15% of soluble label is free fucose; however, 12% was due to degradation during purification. The difference cannot be recycled internally through GDP-fucose; otherwise, this pool would never equilibrate with label in the medium.

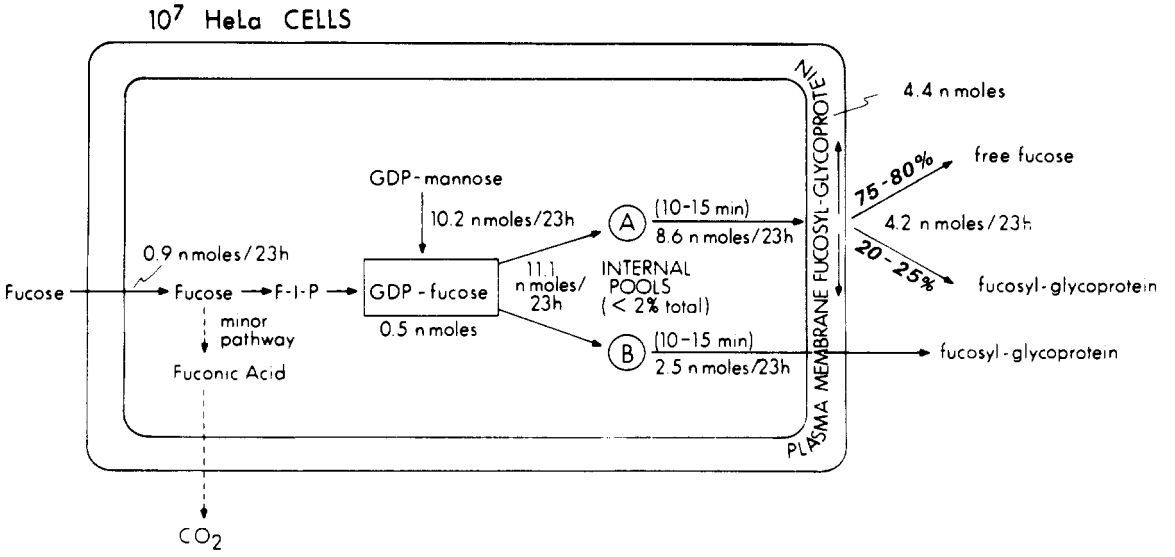

FIGURE 7: Summary diagram. A summary of the data developing from this study is presented here; directly from Figure 1 is calculated the flow rate of label into GDP-fucose and into glycoprotein pools. Since the GDP-fucose specific activity is known, the flow rates into glycoprotein pools can be determined (11.1 nmol 23 h^{-1} (10^7 cells)$^{-1}$). From the observed flow rate of label into whole-cell glycoprotein, it can be determined that actually 8.6 nmol of glycoprotein fucose flows into the cells per generation. 2.5 nmol 23 h^{-1} (10^7 cells)$^{-1}$ is the flow rate of the glycoprotein (Figure 6) released from a small internal pool ("B") directly to the medium without mixing with the bulk cell fucosyl glycoprotein. The cells contain 4.4 nmol/10^7 cells of glycoprotein fucose, as determined by direct measurement. By the arguments already discussed, the measurably significant proportion of this must be in the plasma membrane; hence, of the observed 8.6 nmol of fucose incorporated into plasma membrane glycoprotein in 23 h, 4.2 nmol of it must be turned over. Direct observation of material in the medium characterized 75–80% of this as free fucose and 20–25% as glycopeptide or glycoprotein fucose. Finally, the ratio of measured specific radioactivities of fucose in the medium to that in the GDP-fucose pool shows that 10.2 nmol 23 h^{-1} (10^7 cells)$^{-1}$ of fucose bound to GDP comes from an endogenous source, while 0.9 nmol comes from the medium. The pathway of fucose to CO_2 is minor because no net loss of ^{14}C label from HeLa is seen (Figure 5 and Kaufman and Ginsburg, 1961).

sites had a surface half-time of 5 h and were then internalized, degraded, and ouabain released to the medium with a half-time of 50 h. If ouabain denotes the degradative behavior of its ATPase-binding site, then neither the rate of turnover from the cell surface nor the degradative release into the medium is similar to fucosyl glycoprotein leading to the conclusion that different HeLa S_3 cell-surface species have widely different residency times and degradative pathways. Species are different in their assembly behavior too, since proteins and glycoproteins of HeLa S_3 cells (Atkinson, 1975) and vesicular stomatitis virus infected HeLa cells (Atkinson et al., 1976) do not assemble into membrane (and probably plasma membrane) in a single-step process and probably follow different pathways into the cell surface.

Fucosyl glycoprotein (2.5 nmol) (with the specific activity near that of the nucleotide sugar, see Figure 6) is released directly into the growth medium without mixing with plasma membrane fucosyl glycoprotein for every 4.2 nmol of fucose-labeled material released from the cell surface. If macromolecular fucose from both sources were hydrolyzed to yield the same percent of free fucose, then the measured percent of labeled fucose (as compared to total labeled material) would be expected to remain constant regardless of chase time. In fact, from 2 to 8 h of chase the proportion of free fucose of all labeled material in the medium rose from 48 to 75%. After 2-h chase almost all of the labeled material accumulating in the growth medium will be derived from the cell surface, since the other source of medium glycoprotein fucose would follow GDP-fucose pool which rapidly (75% in 90 min) deequilibrates. Hence, it was concluded that only the cell-surface glycoprotein fucose is hydrolyzed to release free fucose.

Origins of Macromolecular Fucose Appearing in the Growth Medium After Release from HeLa Cells. A substantial part of the macromolecular fucose-labeled material released into the growth medium does not mix with the plasma membrane glycoprotein and approaches the higher specific radioactivity of GDP-fucose. This internal glycoprotein ("B pool", Figure 7) is distinct from another ("A pool") supplying glycoprotein to the plasma membrane. The specific radioactivity of the labeled material appearing in growth medium is consistent with that expected for extracellular glycoprotein deriving from a low and high specific radioactivity source, as shown in Figure 7. An alternate explanation of its high specific radioactivity is that all of the glycoprotein fucose appearing in medium derives from a single cell-surface glycoprotein pool at a specific radioactivity five times (Figure 6) higher than that of the average cell-surface glycoprotein fucose. This possibility is made unlikely because the cell surface has no significant subclass of glycoprotein fucose turning over at a different rate from the average value as indicated by the monophasic label-decay pattern (Figure 4).

Fucosyl glycoprotein released directly to the medium accumulates with the same lag time as fucosyl glycoprotein assembled into the plasma membrane (Figure 1A). The present and previous studies (Atkinson, 1975) have established this lag time at about 10–15 min. If the directly released glycoprotein does not further accumulate intracellularly, it would represent about 0.5% of the total cell fucosyl glycoprotein.

The conclusions drawn in this paper are summarized in Figure 7. GDP-fucose feeds two small internal glycoprotein fucose pools. From one pool is derived the plasma-membrane fucose which eventually enters growth medium, mostly as free fucose. From the other (possibly the same pool) is derived macromolecular fucose which bypasses the cell surface to enter growth medium.

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Differential Effects of Puromycin on the Incorporation of Precursors of Rhodopsin in Bovine Retina†

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ABSTRACT: Bovine retinas incubated in vitro sustain the synthesis of opsin and rhodopsin as monitored by the incorporation of labeled leucine, mannose, and glucosamine. Puromycin, an inhibitor of protein synthesis, effectively blocks the incorporation of leucine and mannose into opsin and rhodopsin of rod outer segments. However, the incorporation of glucosamine into opsin and rhodopsin is not immediately blocked. Instead, it continues for a time suggesting not only core oligosaccharide synthesis but also the secondary glycosylation of a pool of preformed opsin which is thought to be transiently accumulated in the photoreceptor Golgi complex. Galactose, not normally found in rhodopsin, is also incorporated into both opsin and rhodopsin. This incorporation appears to be competely insensitive to puromycin, suggesting that it may occur in the rod outer segments involving only preexisting glycoproteins.

The oligosaccharide component of rhodopsin consists of two types of sugar residues, mannose and *N*-acetylglucosamine (Heller, 1968; Heller and Lawrence, 1970; Plantner and Kean, 1976; Shichi et al., 1969). These sugars occur in ratios of approximately 9 mannose and 5 *N*-acetylglucosamine residues per mol of rhodopsin (Plantner and Kean, 1976) with *N*-acetylglucosamine acting as the link between the oligosaccharide and an asparagine residue of the polypeptide (Heller and Lawrence, 1970). Oligosaccharides of similar composition are found in many glycoproteins and can have a variety of structures based on the *N*-acetylglucosamine linkage to asparagine (Montgomery, 1972). These oligosaccharides are referred to as core oligosaccharides since they can serve as the base upon which more complex carbohydrate chains are assembled. The synthesis of core oligosaccharides takes place on the rough endoplasmic reticulum (Bouchilloux et al., 1970) probably as a lipid-oligosaccharide complex with the oligosaccharide ultimately being transferred to newly synthesized polypeptides (Lennarz, 1975). Puromycin inhibits protein synthesis in the rough endoplasmic reticulum and should cause a substantial inhibition of the incorporation of core oligosaccharides since the acceptor polypeptide would not be produced. Puromycin has been shown to block the incorporation of leucine into rhodopsin in both bovine (O'Brien et al., 1972) and frog retinas (Basinger and Hall, 1973). The oligosaccharide of rhodopsin has the simple composition of a core oligosaccharide and may be synthesized through a lipid-linked intermediate (Kean and Plantner, 1976). If rhodopsin has only a core oligosaccharide, then puromycin should substantially

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