Synthesis and Assembly of HeLa Cell Plasma Membrane Glycoproteins and Proteins*

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PAUL H. ATKINSON

From the Departments of Pathology, Developmental Biology and Cancer, Albert Einstein College of Medicine, Bronx, New York 10461

SUMMARY

At least 60% of the fucose residues in HeLa cell glycoprotein are nonreducing, terminal, and closely proximal to the protein carbohydrate linkage. As determined by pulse-labeling with [3H]fucose and sizing glycopeptides in Sephadex chromatography these residues are added near the time of completion of oligosaccharide chains. Glycoproteins, the large bulk if not the only macromolecules labeled with radioactive fucose in HeLa cells, were not soluble in ethanol or chloroform-methanol, 2:1, but were substantially solubilized by chloroform-methanol-water, 10:10:3. Folch extraction of labeled cells and analysis of the upper phase revealed little if any [3H]fucose-labeled glycosphingolipids. Studies on the distribution of radioactively labeled glycoprotein in various cell fractions show that in uniform labeling conditions fucosylated glycoproteins accumulate in the plasma membrane specifically. Pulse-chase and protein synthesis inhibitor studies show that there is an internal pool of completed fucosylated glycoprotein, taking not less than 12 min to deplete. From this pool newly synthesized glycoprotein moves to the plasma membrane with a transit time of 12 min and little was found soluble in the cell. By contrast, a pool of protein labeled with 14C-aminoacids and precursor to plasma membrane protein is small and depleted almost immediately. From this pool newly synthesized protein molecules move to the plasma membrane with a transit time of less than 2 min. It would appear that these two distinct molecular components of plasma membranes may be assembled into membranes sequentially or into the plasma membrane independently.

The mode of assembly of animal cell membranes is still obscure. One alternative would view the growth of existing membrane structures by fusion with newly "crystallized" membrane whose components were assembled into membrane simultaneously. A second model would view different molecular species being added randomly or sequentially to the growing membrane and would not exclude the individual entry of component molecules in the growth of completed membrane structures, as has been suggested in bacterial membrane synthesis (1). A model allowing individual or sequential entry of at least some component species would make it easier to explain differentiation of some of the physiologic properties of membranes, as discussed in a review by Oxender (2). He suggested that the construction of functional macromolecular complexes may result from random insertion of components followed by their lateral migration. Subsequently, affinities of the various components for each other might result in the formation of stable complexes, as suggested in the formation of junctional complexes by accumulation (3), nucleation (4), or as in a more general hypothesis explaining various membrane mediated phenomena (5). The question of the timing of assembly into the membrane of component species after their synthesis is vital in these important proposals.

There have been a number of studies on the kinetics of assembly of surface membrane protein (6, 7) or glycoprotein (8-11) or both (12-18); for a review see Ref. 19. However, few have studied such kinetics over closely spaced time periods (minutes, rather than hours). It seemed necessary to study these kinetics on such short time scales since it has become evident from the assembly of the membranes of surface maturing viruses (15, 16) that events were occurring in minutes.

Since macromolecules, probably glycoproteins, containing fucose are markedly enriched in various mammalian cell surfaces (8, 10, 20-24) their biosynthesis and kinetics of assembly into surface membranes can be investigated by following the fate of radioactive fucose. Highly purified HeLa cell plasma membranes are known to label with 14C-aminoacids in addition to [3H]fucose (21). The results will show that 14C-labeled polypeptides stop accumulating in plasma membranes almost immediately after the addition of protein synthesis inhibitor, whereas [3H]fucose-labeled glycoprotein continues to accumulate. Thus, in these experiments the protein in glycoprotein must be an insignificant part of the total in plasma membranes labeled with 14C-aminoacids. Therefore, by preparing plasma membranes from cells pulse-labeled with 14C aminoacide and [3H] fucose, the synthesis and assembly of polypeptides and glycoproteins into surface membranes can be followed individually and simultaneously.

Fucose, in glycoprotein from a number of mammalian cells, has a terminal position closely proximal to the protein carbohydate linkage (25-30), and in a cultured human diploid cell (29) it is probably attached to the N-acetylgalactosamine of this linkage as determined from the specificity of endo-β-N-acetylgalactosaminidase D (31). Use of this enzyme in the experiments

* This investigation was supported in part by Research Grants CA 13402 and CA 05576 from the National Institutes of Health and DRG 1204 from the Damon Runyon Memorial Fund.
to be reported that at least 60% of the HeLa cell fucose containing glycoprotein also has fucose in a similar position. Thus, the kinetics of assembly of at least some of the glycoproteins in surface membranes can be studied utilizing glycoprotein radioactively labeled with a sugar whose position in the oligosaccharide chain is known. The comparative timing of plasma membrane protein and glycoprotein assembly into the plasma membrane of HeLa cells was examined in this study.

MATERIALS AND METHODS

Cells and Radioactive Labeling—HeLa E2 cells were grown in suspension culture as previously described (21). Stock cultures were tested once a week for mycoplasma contamination (32). For long labeling periods (more than 3 hours) cells were labeled in their normal growth density range (1.5 X 10^6 cells/ml to approximately 8.0 X 10^6 cells/ml). All culturing or labeling of cells was performed under sterile conditions. For short labeling periods, unless otherwise stated, cells were collected by centrifugation and resuspended in growth medium at 5 to 10 times their previous density. This results in an economy of isotope utilization but apparently does not affect the linear incorporation of isotope (10, 21).

[^1]


Cytometric and Radioactivity Assays—Assays of 5% trichloroacetic acid-insoluble radioactivity utilizing glass filters were as previously described (21).

Folch Procedure (34) for Lipid Analysis—Chloroform (CHCl3)-methanol (CH3OH) (1:2, v:v) was mixed with 0.1 ml of either homogenized cells or purified neutrophils in 10 ml Tris, pH 8.0, and was homogenized. The mixture was centrifuged at 12,000 X g for 15 min to collect precipitated material. The supernatant was filtered through a Whatman glass fiber paper filter and the nonfilterable radioactivity was recovered. To the filtrate, 1/2 volume (0.4 ml) of water was added and the mixture was shaken. The upper phase was removed and the same volume (about 1 ml) of 0.1 M Tris, pH 7.4, was added and the mixture was shaken. The upper phase was again removed and pooled with the previous upper phase giving final volumes: upper phase, 2.0 ml, lower phase, 1.8 ml. Aliquots were analyzed for radioactivity. The combined upper phase was added to 1 mg/ml of cholesterol and dialyzed 30 hours against three changes of 1.000 volumes of water, lyophilized, and resuspended in CHCl3-CH3OH (2:1). Aliquots were utilized for radioactivity assays. The remaining material was extracted twice with hexane and twice with CHCl3 to remove the cholesterol, and aliquots were chromatographed on thin layer Silica Gel G plates with CHCl3-CH3OH-H2O (70:30:4), and the standards: cerebroside, phospholipid, sulfatide, lecithin, and sphingomyelin.

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formic acid-7.5% acetic acid) or at pH 6.5 (pyridine-acetic acid-
7.5% acetic acid) or at pH 6.5 (pyridine-acetic acid-
latter, [Wlacetyl-Asn-GlcNAc molecular weight 379.)

Other materials used to standardize the G-25 columns so that ap-
proximate molecular weights could be determined were: H-2 allo-
glycosidase digests as markers and the sample solution was applied
to a column of Sephadex G-25, fine (0.9 X 140 cm), which was equil-
ibrated and eluted with 50 mM ammonium acetate, pH 7.0, unless
otherwise specified. Fractions of 1.25 ml were collected and ali-
quots were analyzed by the phenol sulfuric acid method (39) to de-
terminate the position of the standard markers, 
acetylated and stained standards.

Analysis of Products of Glycosidase Digestion by Sephade
 Column Chromatography—Blue dextran (0.2 mg) (blue dextran
2000, Pharmacia, Sweden) and 1 mg each of stachyose (B grade,
Calbiochem) and fucose (Sigma Chemical Co.) were added to the
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RESULTS

Rate of Accumulation of Newly Synthesized Poly
peptides and Glycoproteins in Cells and Membranes—Cultures were doubly
labeled with 
C-amino acids and [3H]fucose. The incorporated
(trichloroacetic acid-precipitable) radioactivity in the unfrac-
tionated cells and in the membrane preparations was determin-
ed to observe the distribution of newly synthesized glycoproteins
and polypeptides between whole cells and plasma membranes
as a function of time after synthesis.

Polypeptides—Accumulation of newly synthesized polypeptide
in the plasma membranes expressed as radioactivity per µg of
protein (Fig. 1A) or recovery per cell or membrane ghost (Fig.
1B) proceeded with little lag. It can be determined by extrapol-
ating to zero incorporation from the more linear portions of
the curves that newly synthesized polypeptide appeared in the
plasma membranes with a lag of 1 to 3 min (dotted lines, Fig. 1,
A and 1B). The lag in appearance of newly synthesized polypep-
dide in the unfractionated cells was not easily measurable but
probably not more than 1 min (solid line, Fig. 1B).

FIG. 1. Accumulation of newly synthesized glycoprotein and
protein in cells and plasma membranes. Cells (1000 ml) at a
density of 3.3 X 10^6 ml were collected by centrifugation and resus-
pended in 225 ml of growth medium at 37° to which were added 200
µCi of [14C-amino acids and 2 mCi of [1,5,6-3H]fucose. At labeling
times of 0, 2, 5, 10, 15, 20, 45, and 90 min; 20-ml aliquots were
processed for plasma membrane preparation. Radioactivity and
protein was determined in homogenates and membrane prepara-
tions.

Membrane plasma ghosts were counted and the recoveries
of cell surfaces determined. A, accumulation of protein (14C-
amino acids) in cells (O-O) or membrane ghosts (a-a) per µg of
protein; B, accumulation of protein (14C-amino acids) in cells
(O-O) or membrane ghosts (a-a) per cell or membran
ghost; C, accumulation of glycoprotein ([3H]fucose) in cells
(O-O) or membrane ghosts (a-a) per µg of protein; D, accumu-
lation of glycoprotein ([3H]fucose) in cells (O-O) or membrane
ghosts (a-a) per cell or membrane ghost.

TABLE I

<table>
<thead>
<tr>
<th>Time of labeling</th>
<th>100 mM Tris, pH 9</th>
<th>100 mM NaCl</th>
<th>10 mM Tris, pH 8</th>
</tr>
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<tbody>
<tr>
<td>µg</td>
<td>cpm</td>
<td>cpm/µg protein</td>
<td>cpm</td>
</tr>
<tr>
<td>0</td>
<td>30</td>
<td>0.1</td>
<td>58</td>
</tr>
<tr>
<td>2½</td>
<td>360</td>
<td>1.3</td>
<td>350</td>
</tr>
<tr>
<td>5</td>
<td>870</td>
<td>2.0</td>
<td>900</td>
</tr>
<tr>
<td>7½</td>
<td>1700</td>
<td>6.0</td>
<td>1840</td>
</tr>
<tr>
<td>10</td>
<td>2720</td>
<td>8.0</td>
<td>2720</td>
</tr>
<tr>
<td>20</td>
<td>4890</td>
<td>16.3</td>
<td>5180</td>
</tr>
</tbody>
</table>

*These and other radioactivity figures refer to the quantity of trichloroacetic acid-precipitable radioactivity in plasma mem-
brane preparations after incubation at 37° for 1 hour in the
various solutions.

Glycoproteins—In contrast to the behavior of newly synthe-
sized polypeptides, a clear lag was observed in the accumula-
tion of newly synthesized glycoprotein in plasma membranes, whether
the data were expressed as radioactivity per µg of protein (Fig.
1C) or as recovery of glycoproteins per cell or membrane ghost.
Fig. 2. Longer term accumulation of glycoprotein and protein in cells and plasma membranes, the same as for the legend to Fig. 1 except the time points include 20, 45, and 90 min of labeling. A, accumulation of protein (14C-aminoacids) in cells (O-O) or membrane ghosts (A-A) per μg of protein; B, accumulation of protein (14C-aminoacids) in cells (O-O) or membrane ghosts (A-A) per cell or membrane ghost; C, accumulation of glycoprotein ([3H]fucose) in cells (O-O) and plasma membrane ghosts (A-A) per cell or membrane ghost.

TABLE II

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein</th>
<th>Recovery of 14C-aminoacid-labeled protein</th>
<th>Recovery of 3H-fucose labeled protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>H110</td>
<td>23.8</td>
<td>7.3%</td>
<td>10.0%</td>
</tr>
<tr>
<td>M10</td>
<td>17.6</td>
<td>4.8%</td>
<td>9.7%</td>
</tr>
<tr>
<td>H20</td>
<td>45.4</td>
<td>7.5%</td>
<td>10.9%</td>
</tr>
<tr>
<td>M20</td>
<td>22.3</td>
<td>7.5%</td>
<td>10.7%</td>
</tr>
<tr>
<td>H30</td>
<td>63.2</td>
<td>7.5%</td>
<td>10.7%</td>
</tr>
<tr>
<td>M30</td>
<td>35.1</td>
<td>7.5%</td>
<td>10.7%</td>
</tr>
<tr>
<td>H60</td>
<td>97.5</td>
<td>7.8%</td>
<td>12.0%</td>
</tr>
<tr>
<td>M60</td>
<td>70.5</td>
<td>7.8%</td>
<td>12.0%</td>
</tr>
<tr>
<td>H90</td>
<td>117.4</td>
<td>6.6%</td>
<td>12.0%</td>
</tr>
<tr>
<td>M90</td>
<td>116.9</td>
<td>6.6%</td>
<td>12.0%</td>
</tr>
<tr>
<td>H120</td>
<td>220.5</td>
<td>6.6%</td>
<td>12.0%</td>
</tr>
<tr>
<td>M120</td>
<td>127.2</td>
<td>6.6%</td>
<td>12.0%</td>
</tr>
</tbody>
</table>

* Cells were grown from a density of 18 × 10⁴/ml to a density of 38 × 10⁴/ml in the presence of 0.1 μCi/ml of [14C]fucose over a time of 19 hours.
* The characteristics of the sucrose gradient used were described in detail elsewhere (10), however, the 500 × g-min supernatant contained the plasma membrane ghosts, which were purified and recovered after zonal centrifugation in Fraction IV.
* The recovery of plasma membrane ghosts was 21.9%, thus the corrected recovery of glycoprotein was 07.6%.
* The recovery of plasma membrane ghosts was 11.6%, thus the corrected recovery of radioactive glycoprotein was 69.8%, and protein was 89%.

(FIG. 1D). Longer term time points were taken in this same experiment in order to extrapolate to zero incorporation when more linear rates of accumulation prevailed in the membranes. It can thus be determined that newly synthesized glycoprotein appeared in the plasma membranes with a lag of about 20 min (dotted lines, Fig. 2, C and D). On the other hand, newly synthesized glycoprotein appeared in the whole cells with a lag of about 5 min (solid lines, Fig. 1, C and D). Therefore, the time for newly synthesized fucosyl glycoprotein to appear in the plasma membrane after its appearance in the cell was about 15 min. That is, the time for newly synthesized molecules to enter an internal pool, mix with pre-existing unlabeled glycoproteins, and then to travel to the plasma membrane was about 15 min. The plasma membranes appeared to be the final cellular site of accumulation of fucosyl glycoprotein because once glycoproteins begin to accumulate in the plasma membranes they increased in specific radioactivity at about 5 times the rate of the cells (Fig. 2C). This conclusion was confirmed by examination of the distribution of fucosyl glycoprotein in various cell fractions after a 19-hour labeling period (Table III). The fractions in the table are described in detail elsewhere (10) and consisted of (a) homogenized cells; (b) homogenate supernatant (homogenate minus nuclei and unruptured cells); (c) nuclei and unruptured cells; (d) zonal centrifugation fractions arising out of purification of plasma membranes from the homogenate supernatant. The highest percentage of fucose-labeled glycoprotein and the maximum specific radioactivity occurred in zonal Fraction IV (10) which contained the purified intact plasma membrane ghosts (Table III). Further purification resulted in an increase in the specific radioactivity to a final value of about 12 times that of the homogenate (Table III). Correcting for the recovery of plasma membranes, about 70% of the cell's total fucose-labeled glycoprotein could be accounted for in the cell surface (Table III). After a 24-hour labeling period, only 15% of the total

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein recovery</th>
<th>Glycoprotein recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>46,762 cpm/μg</td>
<td>100%</td>
</tr>
<tr>
<td>500 × g min supernatant</td>
<td>27,928 cpm/μg</td>
<td>12.7%</td>
</tr>
<tr>
<td>500 × g min pellet</td>
<td>8,052 cpm/μg</td>
<td>7.3%</td>
</tr>
<tr>
<td>Zonal fractionation of 500 × g min supernatant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction I</td>
<td>16,796 cpm/μg</td>
<td>35.9%</td>
</tr>
<tr>
<td>Fraction II</td>
<td>5,940 cpm/μg</td>
<td>12.7%</td>
</tr>
<tr>
<td>Fraction III</td>
<td>3,040 cpm/μg</td>
<td>7.8%</td>
</tr>
<tr>
<td>Fraction IV</td>
<td></td>
<td>4,567 cpm/μg</td>
</tr>
<tr>
<td>Fraction V</td>
<td>1,313 cpm/μg</td>
<td>2.8%</td>
</tr>
</tbody>
</table>

Thus, the correction for recovery of radioactive glycoprotein was 07.6%.

The recovery of plasma membrane ghosts was 11.6%, thus the corrected recovery of radioactive glycoprotein was 69.8%, and protein was 89%.
It is concluded from this section of results that the time for newly synthesized polypeptide to travel from the site of synthesis to the plasma membrane is very small (about 2 min) while the equivalent time for newly synthesized glycoprotein is much longer (about 15 min).

**Time for Internal Glycoprotein to Reach Plasma Membrane**—It is evident from the previous section of results that a pool of fucosyl glycoprotein exists inside the cell, which is not the site of accumulation, and could contain up to 2.0% of the total cell glycoprotein. To determine how quickly newly synthesized molecules move through or from this pool to the plasma membrane, a “pulse-chase” experiment was performed. In such an experiment, when further incorporation of label into glycoprotein was stopped, the subsequent time at which peak radioactivity occurred in the internal pool and in the plasma membranes was determined. Given sufficient excess (40 mM) of fucose added to medium containing radioactive fucose, further incorporation of radioactivity into glycoprotein can be stopped within minutes (Fig. 3). Lower concentrations were less effective (Fig. 3). A culture was pulse labeled 25 min with \(^{3}H\)-fucose, divided in two, and in one part excess fucose (40 mM) was added to the medium. The other part was used as a control determining the unperturbed behavior of fucosyl glycoprotein. Tri-chloroacetic acid-precipitable \(^{3}H\)-fucose radioactivity per mg of protein and also per cell or ghost was determined at various times in the cells and plasma membranes. Similar to the experiment in Fig. 1, this experiment utilizing \(^{3}H\)-fucose alone also showed a 15-min lag in the appearance of newly synthesized fucosyl glycoprotein in plasma membranes after its appearance in the whole cell, though the data are not presented. In the culture receiving excess fucose, incorporation of \(^{3}H\)-fucose into whole cell glycoprotein continued to increase for about 10 min before reaching a plateau (dotted lines, Fig. 4A). The plasma membrane radioactivity continued to increase for about 25 min (Fig. 4B) then reached a plateau. These data probably reflect the relocation of fucose-labeled glycoproteins from inside the cell to the surface. By subtracting the radioactivity in the plasma membranes from the cell total, the radioactivity in the internal glycoprotein pool can be determined. These data can be deduced from Fig. 4 and by assuming the plasma membranes have about 10% of the cell total protein; see Table II.) It was thus determined that radioactivity peaked in the internal glycoprotein pool about 13 min after the start of the chase. Accumulation of radioactivity in the plasma membranes peaked about 12 min later. At this time the bulk (near 100%) of the labeled fucosyl glycoprotein could be accounted for in the plasma membranes (Fig. 4). Therefore the transit time of glycoproteins from the internal pool to the plasma membranes is about 12 min. Thus, much of the lag time between appearance of newly synthesized glycoprotein in cells and the membranes observed in Fig. 1 is the time to transport fucosyl glycoprotein from an internal pool to the plasma membranes.

**Rate of Depletion of Internal Pools Supplying Polypeptides to Plasma Membranes**—An experiment was performed to determine how quickly the internal pool supplying prelabeled polypeptide to the plasma membranes can be depleted after inhibition of protein synthesis. The experiment was performed utilizing \(^{14}C\)-amino acids to label the polypeptides and double labeled with \(^{3}H\)-fucose to observe the comparative accumulation of fucosyl glycoprotein in the plasma membranes under these conditions. Cells were prelabeled 45 min with these precursors, the culture was divided in two, and cycloheximide was added (15 \(\mu\)g/ml) to one of them. The other portion was used as a control. Accumulation in the plasma membranes of polypeptides labeled with \(^{14}C\)-amino acids increased slightly in the first 7½ min, then ceased abruptly (Fig. 5B), whereas accumulation

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*P. D. Yurchenco, and P. H. Atkinson, unpublished.
of glycoprotein labeled with [3H]fucose was not immediately affected by the drug (Fig. 5A). Two other experiments with 21.4-min time intervals up to 71.4 min, and 5-min time intervals up to 15 min after administration of the drug gave similar results (data not presented). The figure shows the accumulation of labeled polypeptides and glycoproteins in only the plasma membranes of control and drug-treated cultures. Table IV shows the accumulation of these radioactive molecules in the unfractionated whole cells. It can be seen that further incorporation of 14C-aminoacid into polypeptides was immediately inhibited after the addition of cycloheximide whereas inhibition of incorporation of [3H]fucose into glycoprotein was 31 and 35% at 30 and 45 min, respectively, after administration of the drug. Because the inhibition of cellular [3H]fucose incorporation was not noticeable until 30 min after the application of cycloheximide it can be concluded that there is a large pool of glycoprotein acceptors for fucose. The comparative inhibition of accumulation of fucosyl glycoprotein in the plasma membranes at these times can be deduced from Fig. 5 and was 11 and 37%, respectively. Therefore, in the first 30 min after the administration of the drug, its effects were considerably less on the continued accumulation of completed fucosyl glycoprotein in the plasma membrane than on the continued addition of [3H]fucose to the glycoprotein acceptors. Were fucose being added directly to glycoprotein acceptors already in the plasma membranes it would have been expected that the inhibition of accumulation of fucosyl glycoprotein in the plasma membranes matched that in the unfractionated cells (i.e. it would be 31% rather than the observed 11%). This evidence can be taken as confirmation of the conclusion derived from the “pulse” and “pulse-chase” experiments (Figs. 1 and 4, respectively) that there was a pool of fucosylated glycoprotein inside the cell which ultimately accumulated in the plasma membrane. Conversely there seemed only a small, but still measurable pool of protein labeled with 14C-aminoacid because the drug almost immediately prevented further accumulation of prelabeled protein in the plasma membranes (Fig. 5B). The lack of continued accumulation after 71.4 min (Fig. 5B) is taken as further evidence of a quickly depleted pool of protein able to contribute newly synthesized molecules directly, or nearly so (within 2 min, Fig. 1, A and B), to the plasma membranes. Since fucosyl glycoprotein could be observed to continue accumulation up to 45 min after the addition of the drug, whereas 14C-aminoacid-labeled polypeptide did not (Fig. 5), 14C-aminoacid radioactivity in the protein moiety of glycoprotein must be either (a) too small a proportion of the radioactivity in non-glycosylated protein of plasma membranes to affect these data (Fig. 5B), or (b) the cellular pool of protein backbones (acceptors) being fucosylated is so large that it is not significantly labeled with 14C-aminoacids during 45 min of protein synthesis in the prelabeling time. The second alternative seems unlikely because this pool can be significantly depleted (31%, Table IV) in 45 min after the inhibition of protein synthesis by cycloheximide.

Properties of Macromolecules Rapidly Labeled with Radioactive Fucose—In order to interpret more fully the above data on the synthesis and assembly of glycoprotein into plasma membranes, experiments were performed (a) to determine the macromolecular nature of trichloroacetic acid-precipitable material rapidly labeled with radioactive fucose, (b) to determine the position of fucose in the majority of HeLa glycoprotein oligosaccharide chains, and (c) to determine the time, relative to the synthesis of the entire oligosaccharide chain, when fucose is added. The kinetic data on the accumulation of fucose-labeled material were all derived from radioactivity assays involving trichloroacetic acid precipitation. Material in HeLa cells labeled for a period of hours, and containing radioactive fucose, was probably glycoprotein as indicated by its sodium dodecyl sulfate-polyacrylamide gel electrophoretic behavior (21) and lack of extractability with lipid solvents (43). It seemed of importance to this study to know if molecules labeled for very short intervals with [3H]fucose and precipitable with trichloroacetic acid were also glycoprotein.

Precipitable material in an aliquot of homogenates of cells labeled 6, 10, 15, or 60 min with [3H]fucose was collected on filters and washed as outlined under “Materials and Methods” for the assay of radioactivity, and digested exhaustively with pronase.
Analyzed directly for trichloroacetic acid-precipitable radioactivity. Trichloroacetic acid was added. Other 0.1 ml aliquots were analyzed directly for trichloroacetic acid-precipitable radioactivity. The precipitate from the 10 ml aliquots was collected on a glass fiber filter (Whatman, GFA) and washed with 5% trichloroacetic acid and 95% ETOH as outlined under "Materials and Methods" for radioactivity assays, except washing volumes were increased proportionate to this sample size. The filters were placed in a test tube, and 1.0 ml of 10 mM Tris buffer, pH 8, was added and the fucose-labeled glycoprotein on the filter was digested with 6 mg/ml of pronase. The pronase-released material was chromatographed on Sephadex G-25 using blue dextran (excluded), stachyose (MW 666), and fucose (MW 164) as internal markers (arrows, from left, respectively). The paper was cut into 1.5 cm strips, eluted with 1.0 ml of water in scintillation vials, and the radioactivity was assayed. A, pH 1.9; B, pH 6.5.

Other aliquots were precipitated and washed on the filters (Fig. 6). Similar results were obtained with an experiment starting with dialyzed homogenates of cells (instead of acid-precipitable material) labeled 10, 15, 20, and 60 min, except free fucose and fucose-GDP were also eluted when these pronase digests were chromatographed on the Sephadex column. The behavior of the fucose-labeled material pooled from the Sephadex columns (Fig. 6) in high voltage paper electrophoresis at pH 1.9 and pH 6.5 was next observed. At pH 1.9, 60% of the [3H]fucose material labeled 6 min displayed basic characteristics and moved to the negative pole; 18% moved with neutral substances, and 22% moved to the positive pole (Fig. 7A). At pH 6.5 the material either stayed at the origin (36%) or displayed acidic characteristics (64%) (Fig. 7B). This behavior is consistent with the [3H]fucose being bound to material containing amino acids, i.e. consistent with the behavior of glycopeptides (35, 44, 45). Thus, the [3H]fucose material labeled 6 min and more, precipitated by trichloroacetic acid and washed on filters, digested to a soluble form by pronase, and behaving as described in high voltage paper electrophoresis, was probably glycoprotein and contained no free fucose or GDP-fucose. Additional evidence confirming this conclusion is presented below.

Other aliquots of the same homogenates labeled 6, 10, 15, and 60 min with [3H]fucose were (a) precipitated with 5% trichloroacetic acid, collected on filters, and washed with 5% trichloroacetic acid and ethanol as in the standard radioactivity assay (see "Materials and Methods"); (b) treated in exactly the same way except that the filters had a final wash of CHCl₃:CH₃OH 2:1); (c) were analyzed as the first set except that the homogenates were centrifuged at 45,000 rpm in a Spinco SW 50 rotor for 1 hour before sampling. The CHCl₃:CH₃OH did not remove any radioactivity from the 5% trichloroacetic acid-precipitable material and 86 to 95% of the radioactivity was found to be sedimentable (Table V).
The distribution of fucose-labeled material is shown in Table V.

Chloroform-methanol solubility and sedimentability of \([\text{H}]\)fucose-labeled macromolecules

<table>
<thead>
<tr>
<th>Sample*</th>
<th>No treatment</th>
<th>CHCl₃-CH₃OH wash</th>
<th>Nonsedimentable</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cpm</td>
<td></td>
<td>Cpm</td>
</tr>
<tr>
<td>6 min</td>
<td>174</td>
<td>166</td>
<td>24</td>
</tr>
<tr>
<td>10 min</td>
<td>424</td>
<td>373</td>
<td>48</td>
</tr>
<tr>
<td>15 min</td>
<td>601</td>
<td>627</td>
<td>69</td>
</tr>
<tr>
<td>60 min</td>
<td>5312</td>
<td>5140</td>
<td>250</td>
</tr>
</tbody>
</table>

* HeLa cells were labeled for various times with \([\text{H}]\)fucose as described in the legend to Fig. 6.

Bosmann et al. (14) reported that about 25 to 30% of the material in HeLa cells labeled for periods up to 60 min with \([\text{H}]\)fucose was glycolipid. It was investigated in the present study whether a significant amount of \([\text{H}]\)fucose-labeled glycosphingolipid was present at short labeling times. Aliquots of the same homogenates, as in the previous experiments, labeled 6, 10, 15, and 60 min with \([\text{H}]\)fucose were analyzed by Folch extraction. The distribution of fucose-labeled material is shown in Table VI. The upper phase after the Folch extraction was dialyzed in the presence of cholesterol (added to prevent the loss of possible labeled gangliosides) and found to contain significant (20 to 30% of the homogenate) fucose radioactivity (Table VI).

A and B cells were labeled with \([\text{H}]\)fucose as described in the legend to Fig. 6. At 6, 10, 15, and 60 min approximately 10% cells were prepared as for plasma membrane preparation as far as the removal of nuclei step ("Material and Methods").

This material when chromatographed on thin layer silica gel was left at the origin (40, 58, 61, and 83%) or found in the position of free fucose in the plates (13, 25, 22, and 7%), respectively at 6, 10, 15, and 60 min of labeling. When the material was chromatographed on paper in Solvent I, it was found either at the origin or co-chromatographing with free fucose. The material chromatographed with \([\text{I}]\)GDP-fucose on Sephadex G-25 and also on paper in Solvent II.

Chloroform-methanol solubility and sedimentability of \([\text{H}]\)fucose-labeled homogenates

<table>
<thead>
<tr>
<th>Labeling time</th>
<th>([\text{H}])Fucose radioactivity of whole homogenate recovered:</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>Protein</td>
</tr>
<tr>
<td>0</td>
<td>12.1</td>
</tr>
<tr>
<td>10</td>
<td>17.2</td>
</tr>
<tr>
<td>15</td>
<td>11.9</td>
</tr>
<tr>
<td>60</td>
<td>35.6</td>
</tr>
</tbody>
</table>

* Cells were labeled with \([\text{H}]\)fucose as described in the legend to Fig. 6. At 6, 10, 15, and 60 min approximately 10% cells were prepared as for plasma membrane preparation as far as the removal of nuclei step ("Material and Methods").

Chloroform-methanol solubility and sedimentability of \([\text{H}]\)fucose-labeled homogenates

Acetic acid-insoluble material in other lipid solvents HeLa cells were pulse-labeled for 30 min with \([\text{I}]\)fucose. Aliquots of the homogenized cells were precipitated as usual with 5% trichloroacetic acid and the precipitates were collected on filters. The filters were then washed with ethanol or CHCl₃-CH₃OH (2:1), or CHCl₃-CH₂OH-H₂O (10:10:3) (46). There was again no difference in the amount of radioactivity remaining on the filters when washed either with ethanol or CHCl₃-CH₂OH (2:1). However, when washed with the same volume of CHCl₃-CH₂OH-H₂O (10:10:3), 50% of the radioactivity was solubilized. Repeated washings solubilized up to 90% of the material on the filters. The filtrate was collected, evaporated, and water was added. The material was not significantly soluble in water. Pronase digestion released the fucose-labeled material in soluble form and was excluded in Sephadex G-25 column chromatography. No soluble material corresponding to free fucose or GDP-fucose was observed. In high voltage paper electrophoresis at pH 1.9, 60% of the labeled material excluded from the column moved to the negative pole, 21% moved with or near the neutral substance and 19% was slightly acidic. When this excluded material was subsequently digested with a glycosidase mixture which removes most the sugars from glycopeptide oligosaccharides (see below), about 89% moved to the negative pole with little acidic or neutral material. It was concluded that, consistent with the earlier results, most, if not all, the \([\text{H}]\)fucose-labeled material assayed by trichloroacetic acid precipitation on filters, was glycoprotein in nature, even though much was apparently soluble in CHCl₃-CH₂OH-H₂O. The latter property bears striking similarity to the behavior of dolichol-phosphate-sugar compounds (46). However, this latter material in these assays should either be totally insoluble in the aqueous solvents used for chromatography and electrophoresis or else, if decomposed after elution from the trichloroacetic acid precipitate on the filter, should display acidic (anionic) or neutral properties in the electrophoresis system but not basic (cationic) properties.

It is concluded from this section of the results that material short term labeled with \([\text{H}]\)fucose and assayed by trichloroacetic acid precipitation on filters is substantially glycoprotein. Fur-
the cell before appearing in the plasma membrane (Fig. 1) are already substantially complete in the oligosaccharide moiety.

6 min. Whichever interpretation applies it is apparent that alternatively the rate of synthesis of completed oligosaccharide glycoproteins are complete or nearly so when fucose is added, or chains from a possible 6 sugar species is very much faster than 6 sugar residues in such a species. However, because the bulk of fucosyl glycopeptide even at 6 min of labeling was not in such included that much of the oligosaccharide chains of HeLa cell residue has a molecular weight of 180 then there would be about 1140 in molecular weight. Assuming an average hexose amino acid molecular weight is 120) then the oligosaccharide species of 1500 molecular weight must contribute small species and appeared substantially complete it can be concluded that much of the oligosaccharide chains of HeLa cell glycoproteins are complete or nearly so when fucose is added, or alternatively the rate of synthesis of completed oligosaccharide chains from a possible 6 sugar species is very much faster than 6 min. Whichever interpretation applies it is apparent that glycoproteins labeled with [3H]fucose for 6 min and appearing in the cell before appearing in the plasma membrane (Fig. 1) are already substantially complete in the oligosaccharide moiety.

Position of Fucose in HeLa Cell Carbohydrate Chains—Fucose, in glycoprotein from a number of mammalian cells, has a terminal position closely proximal to the protein carbohydrate linkage (29-30). HeLa cell fucose-labeled glycoprotein was tested to determine whether fucosyl residues were in this region also. This was done by use of a mixture of glycosidases (neuraminidase, β-galactosidase, β-N-acetylgalactosaminidase, and endo-β-N-acetylgalactosaminidase D) all of which are known to be active with various (including) mammalian glycoprotein heterosaccharides. In particular, endo-β-N-acetylgalactosaminidase D is known to cleave between the N-acetylgalactosamine residue attached to asparagine and the second N-acetylgalactosamine of the oligosaccharide chain of the ovalbumin glycopeptide Asn-(GlcNAc)z-(Man)n (31). It is highly likely the same mechanism applies to various mammalian glycopeptides also digested with this enzyme (27-29). As noted above, the bulk of HeLa cell glycopeptides, long term labeled, have a molecular weight of around 3000 and are substantially excluded from a Sephadex G-25 column. However, after glycosidase digestion about 60% of the glycopeptides were reduced to fucose-containing fragments of approximately 700 molecular weight which were found included in the column (Fig. 8A). These fragments still appeared to contain amino acids, i.e. were glycopeptides as deduced from their behavior in high voltage paper electrophoresis at pH 1.9 and pH 6.5 (data not shown, cf. Fig. 7). Mild acid hydrolysis released from this glycopeptide fragment 89% of the label which was characterized as fucose by paper chromatography in Solvent 1. Thus, the fucose residues in these fragments are probably nonreducing, terminal. The 40% of material remaining undigested by the glycosidase mixture was pooled and upon redigestion and chromatography was not further digested as shown by absence of significant quantities of glycopeptide of about 700 molecular weight (Fig. 8B). This material was then treated with α-mannosidase and further treated with glycosidase mixture and again, there was no further digestion (Fig. 8C). Since the glycosidase mixture reduced 60% of HeLa cell fucose-labeled glycopeptides to a heterogenous glycopeptide fragment of about 700 molecular weight (Fig. 7A) it is therefore big enough to contain only 2 to 3 amino acids and 2 to 3 sugars. Therefore, at least 60% of fucose residues in HeLa cell glycoprotein are probably terminally located not more than 1 or 2 sugar residues from the peptide backbone. In the pulse-labeling study (Fig. 6), some glycopeptide intermediaries of 700 molecular weight might have been ex-

![Fig. 8. Sephadex G-25 chromatography of the products of glycosidase digestion.](http://www.jbc.org/)

**Fig. 8.** Sephadex G-25 chromatography of the products of glycosidase digestion. Approximately 2 X 10⁷ cells labeled 60 min with 20 μCi/ml of [3H]fucose were processed as for preparation of plasma membranes. The denucleated cytoplast was then dialyzed 1 day against 1000 volumes and two changes of water at 4°, and was digested with pronase. The glycopeptides were prepared by Sephadex G-50 chromatography and digested with a mixture of glycosidases (endo-β-N-acetylgalactosaminidase D, β-N-acetylgalactosaminidase, β-galactosidase, neuraminidase), and the digestion products were chromatographed on Sephadex G-25. A, glycosidase digested glycopeptides; B, pooled Fractions 37 to 46 (Fig. 7A) redigested with the glycosidase mixture and rechromatographed; C, pooled Fractions 37 to 50 (Fig. 7B) digested with α-mannosidase, redigested with the glycosidase mixture and rechromatographed. Arrows denote the peak elution positions of blue dextran (excluded), stachyose (MW 660), and fucose (MW 104), respectively, from the left of the diagrams.
pected if fucose is added during synthesis of the oligosaccharide chains in the sequence suggested by its relative proximity to the protein-carbohydrate linkage. The fact this intermediate was not observed means fucose in at least 60% of the glycoprotein is not added early in the synthesis of oligosaccharide chains, confirming the conclusion derived from Fig. 6.

**DISCUSSION**

It is concluded from the data presented (Fig. 1, 2, and 4) that glycoproteins, fucosylated inside the cell, and subsequently accumulated in the surface membrane, move from an internal pool (the site of synthesis) to the site of accumulation in about 12 min (transit time), confirming previous preliminary results (10). An internal site of synthesis of HeLa cell glycoprotein with a subsequent migration to the plasma membrane agrees with previous studies (14) though these have been extended because the kinetics are now defined in minutes. Confirming that this lag is maintained even when more uniform labeling conditions applied, prelabeled glycoproteins can be “chased” into the plasma membrane (Fig. 4) after excess unlabeled fucose was added to a culture incorporating radioactive fucose. The “chase” as performed in these studies is not a “chase” in the sense that all labeled molecules move en bloc from one pool to another. Rather, the cessation of labeling caused by 40 mM fucose in the medium is probably due in part to expansion (and therefore dilution of label) of the GDP-fucose pool known to occur when fucose is added to the medium of HeLa cells (38) as well as to the chasing of the labeled molecules. The pool of fucosylated glycoprotein possibly also expands as more fucose is added to the medium. This latter possibility derives from the data in Fig. 3 where it is shown that the reduction in incorporation of [H]fucose into glycoprotein was substantially less than that which would be predicted from the reduction in specific activity of the [H]fucose label. Thus, addition of limited amounts of fucose to the medium may also cause stimulation of fucosylation with a concomitant expansion of the fucosyl glycoprotein pool. Such a stimulation would be expected if substrate (fucose in the medium) was limiting for a process (fucosylation of glycoprotein) whose over-all K_m is high. It is also known that mammalian cell GDP-fucose can be derived from two sources: namely, exogenous fucose (38) and an endogenous source, probably GDP-mannose (47). We have confirmed the conclusion by direct measurement of the specific radioactivity of GDP-fucose in the GDP-fucose pool; when equilibrated with 0.4 mM [H]fucose in the medium, 12 to 17 fucose molecules of the pool were derived from endogenous sources to every 1 from the medium. Thus it is also possible that fucose added to the medium changes the relative contributions from these sources giving the appearance of increased [H]fucose incorporation into glycoprotein as its specific activity is decreased. Whenever two of these explanations or combination thereof apply to the data in Fig. 3, the fact that the GDP-fucose pool expands in response to added fucose in the medium does not affect the estimate of the time to transport molecules from a subsequent internal glycoprotein pool into the plasma membrane. This is because the “chase” was effective in chasing labeled molecules from the soluble pools to the internal glycoprotein pool into the plasma membrane. Thus, the time between maximum radioactivity in the internal glycoprotein pool and that in the plasma membranes could be measured and was about 12 min. However, the plasma membranes reached maximum radioactivity 25 min after the chase was started. Thus 13 min of this time has to do with equilibration of pools precursor to the internal glycoprotein pool and need not be considered in the transit time estimate. As had been observed in a previous study (10), much, if not all the labeled glycoprotein could be accounted for in the plasma membranes at the end of the chase period. The possibility that the internal fucosyl glycoprotein pool may also expand in response to added fucose in the medium apparently does not significantly affect the estimate of transit time since the plasma membranes were observed to continue labeling at the same rate as controls for nearly 25 min after the start of the chase followed by a relatively sudden plateauing as the labeled contents of the internal pool ceased accumulation in the membrane (Fig. 4B). This kinetic behavior is more consistent with the chasing of almost the entire contents of the internal pool (confirming previous data (10)) before possible dilution and expansion of the internal glycoprotein pool became significant. Thus, the transit time of fucosyl glycoprotein from an internal pool to the plasma membrane was about 12 min and may be made up of several steps in which the oligosaccharide chain of glycoproteins was completed by the addition of other hexoses after the addition of fucose.

Labeled glycoprotein in all of these possible steps would of course be trichloroacetic acid-precipitable and thus in these studies would resemble the behavior of completed glycoproteins. However, it can be concluded that the bulk of the oligosaccharide chains even at the shortest labeling time tested (5 min) must have been completed or nearly so before fucose was added. Thus the transit time of completed fucosyl glycoproteins from an internal pool to the plasma membranes must be greater than 6 (6 to 12) min.

Nothing in these studies rules out the participation of a possible fucosyl-phosphoryl polypropenol intermediate similar to other sugar phosphoryl polypropenol compounds suggested to be involved in the synthesis of some glycoproteins oligosaccharides (40, 48, 49). Such a compound if similar in properties to dolichol-glycosylated-glycose compound described (46) would probably be precipitable with trichloroacetic acid and thus resemble, in radioactivity assays, completed fucosyl glycoprotein. Much of this glycoprotein appeared soluble in CHCl_3-CH_3OH-H_2O (10:10:3), and in this respect resembles dolichol-glycosylated-hexose (46), but was also similar to the solubilization of membrane glycoproteins from mammalian erythrocytes with CHCl_3-CH_3OH (50). However, the bulk of the internal rapidly labeled trichloroacetic acid-precipitable material was probably already bound to glycoproteins as determined by the behavior of their pronase-released glycopeptides in high voltage paper electrophoresis. No intermediary or growing form of glycopeptide containing only 2 to 3 sugars and consonant with the position of fucose proximal to the protein-carbohydrate linkage was observed by pulse-labeling with [H]fucose. Thus either the fucosyl residues were added along with other sugars as an oligosaccharide via a dolichol intermediate (46, 48, 49) or else they were added after the bulk, if not all, of the other sugars had been added.

The results and conclusions derived for the behavior of [H]-fucose-labeled glycoproteins in HeLa cells by the present methods are in close agreement with those of an autoradiographic study of the behavior of such glycoproteins studied in vivo in rats (8, 9). The autoradiographic results have been extended because the comparative behavior of non-glycosylated proteins have also been studied. By use of endo-β-N-acetylglucosaminidase D (31) in the glycosidase mixture used to degrade glycopeptides, a fragment was obtained which, from the known specificity of the enzyme and from the molecular weight of the
fragment, should contain only the N-acetylglucosamine of the protein-carbohydrate linkage fucose and 2 to 3 amino acids. A larger fucosyl glycopeptide, apparently not digested with the endoglycosidase, was also observed after the use of the glycosidase mixture and could not be reduced further in size by a combination of neuraminidase, β-N-acetylglucosaminidase, α mannosidase, and endo-β-N-acetylglucosaminidase D. The reason for this is not clear though it may be related to the presence of mannos-containing glycopeptides of the thyroglobulin Unit A type which seem resistant to endo-β-N-acetylglucosaminidase D (31). However, the HeLa resistant glycopeptides contain fucose; the thyroglobulin Unit A glycopeptides do not (51). It is of interest to note the presence of such resistant glycopeptide species in the human cancer cell (HeLa) where little if any fucose containing glycopeptides in the normal cell was resistant to this enzyme mixture (29).

In marked contrast to the time it takes to transport newly synthesized fucosyl glycoprotein (12 min) or completed fucosyl glycoprotein (more than 6 min) from an internal pool to the plasma membrane, the equivalent time taken to transport newly synthesized polypeptides is not more than 2 min. Furthermore, the internal pool of polypeptides is small because it is depleted in not more than 7½ min in contrast to the fucosyl glycoprotein pool which is depleted in not less than 12 min. If fucosyl glycoproteins and polypeptides are then assembled as a membrane simultaneously from their internal pools of completed molecules together with other membrane components such as phospholipid, it must take not more than 2 min for the entire assemblage to enter or fuse with the plasma membrane. This is impossible because the time necessary for the newly synthesized or completed fucosyl glycoproteins to leave their internal pool and enter the plasma membranes is more than 6 min. The difference in pool size also suggests that the pools are behaving independently of each other. Thus it seems that at one stage after their completion, plasma membrane proteins and glycoproteins are not physically anchored or bound together and for at least 4 min of their greater than 6-min journey to the plasma membranes the glycoproteins are not accompanied by the membrane polypeptides. This conclusion, taken together with the sedimentation data which show much of the fucosyl glycoprotein to be particle-bound and perhaps membrane-bound in cell homogenates even at 6 min of labelling time would seem to support a model where membrane components are added sequentially and is in general agreement with one favored to explain the heterogeneity of degradation of the proteins of rat liver membranes (52) and other membrane turnover data (19). It is suggested, in view of the very small pool of newly synthesized polypeptides, that the nonglycosylated proteins may be added directly to the plasma membranes, rather than being added to an internal piece of membrane on its way to fusing with the plasma membrane.

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Synthesis and assembly of HeLa cell plasma membrane glycoproteins and proteins.
P H Atkinson


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