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

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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 3Hfucose (21). The results will show that 14C-labeled polypeptides stop accumulating in plasma membranes almost immediately after the addition of protein synthesis inhibitor, whereas 3Hfucose-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 aminoacid 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 carbohydrate 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...
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 × 10^6 cells/ml to approximately 8.0 × 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 excess of isotope utilization but apparently does not affect the linear incorporation of isotope (10, 21). 3H-Aminoacid mixture (15 amino acids, 80 to 400 mCi/mmol) was used at concentrations of 1 to 20 μCi/ml. L-[1-3H]fucose (13.4 Ci/mmol) or, if so stated L-[1,5,6-3H]fucose (4.8 Ci/mmol) was used at concentrations of 0.1 to 1.0 μCi/ml. L-[β-3H]mannose (10 Ci/mmol) was used at concentrations of 1 to 20 μCi/ml. L-[1-14C]fucose (4.8 Ci/mmol) was used at concentrations of 1 to 20 μCi/ml. L-[1-14C]fucose (50.8 mCi/mmol) was used at concentrations of 0.1 μCi/ml. All these compounds were obtained from New England Nuclear.

GDP-[L-14C]fucose, obtained from Amersham-Searle Corp. as guanosine diphosphate[L-fucose-14C(U)], ammonium salt, was used as a tracer.

Processing of Radioactively Labelled Cells—Cells (in 10 to 20-ml aliquots) were transferred from radioactively labeled cultures, at designated intervals, into 40-ml centrifuge tubes containing about 20 ml of frozen, crushed Earle's (33) solution. The tube was topped off with ice-cold Earle's solution and the cells were sedimented to the pellet. The cells were washed twice more with 40 ml of ice-cold Earle's solution. The final washed pellet of cells was stored on ice prior to further processing.

Preparation of Plasma Membranes—The method for purification of HeLa cell plasma membranes has been described previously (10, 21). The ghosts were resuspended for analysis after one sucrose gradient separation (21). This procedure is referred to as once-cycled (21) plasma membranes. Particulate recovery was determined by counting the intact ghosts in a hemacytometer (10, 24). An extensive light and electron microscopic examination of the particulate composition of the plasma membrane fraction of this degree of purity will be described elsewhere.1

Such fractions were shown to be substantially free of unruptured whole cells, nuclei, and mitochondria. Some (very little) rough endoplasmic reticulum was present.

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) (1.7 ml, 2:1) was mixed with 0.1 ml of either homogenized cells or purified neutrophils in 10 μl Tris, pH 8.0, and was homogenized. The mixture was centrifuged at 12,000 × 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 1.0 ml) of CHCl₃–CH₃OH–H₂O (3:18:47) 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 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 of this were used for radioactivity.

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 in CHCl₃–CH₃OH–H₂O (70:30:4) using the standards: cerebroside, phospholipid, saflacin, lecithin, and sphingomyelin.

Pronase Digestion—Fucose-labeled homogenized HeLa cells or plasma membranes were digested with 6 mg/ml of pronase (B grade, Calbiochem) in 250 μl Tris-HCl buffer, pH 8.0-10 μl CaCl₂. The reaction mixture plus a small amount of toluene was incubated at 37° for 24 to 36 hours, then another 6 mg/ml of pronase were added and the digestion continued for another 24 hours. Precipitated material was removed by low speed centrifugation.

Preparation of Glycopeptides by Sephadex G-50 Chromatography—Glycopeptides were prepared from pronase digests by chromatography on Sephadex G-50, 2.5×50 cm, as previously described (29). No fucose-labeled material was excluded. There were two included peaks of radioactivity. One eluted near the excluded volume and had about 25% of the radioactivity. A second peak was heterogeneously distributed around 3000 in molecular weight and had about 75% of the radioactivity. The lower molecular weight peak containing the bulk of the included material was pooled, lyophilized, and resuspended in distilled water before further analyses.

Glycosidase Digestion—Glycosidase activities, unless otherwise specified, were measured by hydrolysis of the appropriate p-nitrophenyl pyranoside (35, 36). A unit of enzyme activity is defined as 1 μmol of p-nitrophenol released per min. Endoglycosidase activity was measured as ability to degrade fucose and mannose-labeled glycopeptides to glycopeptides containing fucose and oligosaccharides containing mannose (29). Neuraminidase (Vibrio cholerae) was obtained from General Biochemicals, Chagrin Falls, Ohio. One milliliter of the enzyme is described as able to release 500 μg of N-acetylneuraminic acid from a glycoprotein substrate when incubated at 37° for 15 min.

β-Galactosidase was prepared from the culture fluid in which Disgacoccus pneumoniae was grown according to the procedure of Hughes and Jeanloz (36) as previously described (29). Endo-β-N-acetylglucosaminidase D (31) co-purified with β-N-acetylglucosaminidase after ammonium sulphate precipitation, Sephadex G-150 column chromatography, and DEAE-Sephadex A-25 chromatography as previously described (29). The purified, partially purified, and free mass of fucose from labeled glycopeptides. The neuraminidase, likewise, did not release free fucose (29). Proteolytic activities in similar preparations of these enzymes, as determined by the release of labeled amino acids from mouse myeloma immunoglobulin G, were not detected (28).

Fucose-labeled glycopeptides in water (200 μl) were incubated at 37° for 15 hours with 200 μl of 200 mM citrate-phosphate buffer, pH 6; 200 μl of β-N-acetylglucosaminidase (0.56 unit/ml; 0.22 unit/mg of protein) which also contained the endo-β-N-acetylglucosaminidase D; 200 μl of β-galactosidase (0.09 unit/ml; 0.03 unit/mg of protein); 200 μl of neuraminidase; 200 μl of 1 2 mM NaCl and an overlay of toluene. Neuraminidase and β-galactosidase were always included in the reaction mixture because of the high degree of endo-β-N-acetylglucosaminidase hydrolysis in their presence (20, 21). A partially purified preparation of α-mannosidase, prepared from Jack Bean meal (Sigma Chemical Co.) according to the procedure of Snait and Levery (37), a generous gift from Dr. Takashi Muramatsu, Kobe University School of Medicine, Kobe, Japan, contained 234 units of enzyme per ml. [3H]Fucose-labeled substrate (200 μl) in water was digested with 200 μl of α-mannosidase in 100 mM phosphate buffer, pH 6.5 and 50 μl of 15 mM CaCl₂. The reaction mixture was then incubated for 18 hours. Precipitated material was removed by low speed centrifugation.

The remaining material was transferred twice with hexane and twice with CHCl₃ to remove the cholesterol, and aliquots were chromatographed on thin layer Silica Gel G plates in CHCl₃–CH₃OH–H₂O (70:30:4) using the standards: cerebroside, phospholipid, saflacin, lecithin, and sphingomyelin.

Paper Chromatography and Electrophoresis—Descending paper chromatography of samples hydrolyzed and resuspended in water was carried out on Whatman No. 3MM paper in pyridine-ethyl acetate-water (1:3:5.6:1.15), Solvent I (38), or in ethanol-1.0 M ammonium acetate (7:3), Solvent II (38). The time for development was 15 to 20 hours. Paper electrophoresis was carried out on 1.5-inch-wide strips of Whatman No. 1 paper at pH 1.9 (25%}

formic acid-7.5% acetic acid) or at pH 6.5 (pyridine-acetic acid) or at pH 6.5 (pyridine-acetic acid). The hydrolysate was desalted in a Sephadex G-10 column (2.5 × 100 cm), equilibrated, and eluted with water, to which the lyophilized eluate was suspended. The lyophilized eluate was suspended in 100 to 200 µl of water and subjected to paper chromatography in Solvent 1 with radioactive and stained standards.

Analysis of Products of Glycosidase Digestion by Sephadex G-25 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 glycosidase digest as markers and the sample solution was applied to a column of Sephadex G-25, fine (0.9 × 140 cm), which was equilibrated and eluted with 50 mM ammonium acetate, pH 7.0, unless otherwise specified. Fractions of 12.5 ml were collected and aliquots were analyzed by the phenol sulfuric acid method (39) to determine the position of the standard markers, stachyose and fucose. Other materials used to standardize the G-25 columns so that approximate molecular weights could be determined were: H-2 alloaminoacyl-tRNA, molecular weight 2900 (39), a gift from Dr. Stanley Nathenson, Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, N.Y.; unfraccionated ovalbumin glycopeptide, molecular weight 1900 (29), [14C]ethyl-Asn-(GlNAc)2-(Man)3, molecular weight 1993, both generous gifts from Dr. Takashi Muramatsu (Ref. 31) and the endo-β-N-acetylglucosaminidase D glycopeptide product of the latter, [14C]ethyl-Asn-GlNAc molecular weight 379.

Lowry Protein Assay—Protein was determined by the Lowry method (40) as modified by Cuccarini and Eagle (41).

RESULTS

Rate of Accumulation of Newly Synthesized Polypeptides and Glycoproteins in Cells and Membranes—-Cultures were doubly labeled with [14C]aminoacids and [3H]fucose. The incorporated (trichloroacetic acid-precipitable) radioactivity in the unfractionated cells and in the membrane preparations was determined 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 extrapolating 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. 1A and 1B). The lag in appearance of newly synthesized polypeptide in the unfractionated cells was not easily measurable but was probably not more than 1 min (solid line, Fig. 1B). Therefore the time of transport of polypeptides from the site of synthesis to the plasma membrane was about 2 min. Portions of membrane preparations labeled with [14C]aminoacids for various times from 0 to 20 min were treated under conditions in which possibly contaminating aminoacyl-tRNA should be discharged (42). No difference in trichloroacetic acid-precipitable radioactivity was observed between discharging and control conditions (Table I). Thus, the material being labeled with the [14C]aminoacids in the membranes is probably not contaminating aminoacyl-tRNA. In longer labeling periods in the same experiment as in Fig. 1 the radioactivity per µg of protein of plasma membranes labeled with [14C]aminoacids never exceeded that of the cells (Fig. 2A, Table II). The per cent recovery of total cell radioactive polypeptides in the plasma membranes was constant, regardless of the length of labeling time (Fig. 2B, Table II) underscoring the point that the lag in accumulation of newly synthesized polypeptides in the plasma membranes after their appearance in the cells was a very small interval.
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 (\(^{14}\text{C}\)-aminoacids) in cells (○—○) or membrane ghosts (△—△) per \(\mu\text{g}\) of protein; B, accumulation of protein (\(^{14}\text{C}\)-aminoacids) in cells (○—○) or membrane ghosts (△—△) per cell or membrane ghost; C, accumulation of glycoprotein (\({^{3}H}\text{fucose}\)) in cells (○—○) and plasma membrane ghosts (△—△) per \(\mu\text{g}\) of protein; D, accumulation of glycoprotein (\({^{3}H}\text{fucose}\)) in cells (○—○) and plasma membrane ghosts (△—△) per cell or membrane ghost.

### Table II

<table>
<thead>
<tr>
<th>Sample (\times 10^{4}\text{ ml})</th>
<th>Protein (%)</th>
<th>Recovery(^a) of (^{14}\text{C})-aminoacid-labeled protein (%)</th>
<th>Recovery(^b) of protein (%)</th>
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<td>127.2</td>
<td>7.5</td>
<td>12.0</td>
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\(^a\) Cells (1200 ml) at a density of \(34 \times 10^{4}/\text{ml}\) were collected by centrifugation and resuspended in 220 ml. \(^{14}\text{C}\)-aminoacids (240 \(\mu\text{Ci}\)) were added and samples removed for membrane preparation as outlined under "Materials and Methods". \(H\) = homogenate, \(M\) = plasma membranes at 10, 20, 30, 60, 90, and 120 min of labeling.

\(^b\) Corrected for recovery of plasma membrane ghosts, which was 20.6, 18.6, 11.5, 16.0, 10.1, and 10.1% at the respective time points from 10 to 120 min.

(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 plasma membrane after its appearance in the cell was about 15 min. That is, the time for newly synthesized molecules to enter the plasma membrane after its appearance in the cell 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
sary to inhibit further incorporation of radioactive fucose. Cells at a density of $6 \times 10^6$/ml were labeled with 10 $\mu$Ci/ml of [1,5,6-\textsuperscript{3}H]fucose (concentration approximately 2 $\mu$Ci/ml). At various intervals aliquots (2 $\times$ 0.5 ml) were pipetted into 2.0 ml of ice-cold Earle’s solution and centrifuged to a pellet. The cells were lysed by resuspension in 1.0 ml of H$_2$O and 1.0 ml of 10% trichloroacetic acid was added. Radioactivity was assayed as outlined under “Materials and Methods.” At 60 min of labeling (arrow) various portions of the culture were adjusted to 0.02 mM (O--O), 2 mM (O--O), or 40 mM (O--O) unlabeled fucose. One portion contained only radioactive fucose (O--O, 0.002 mM).

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 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.

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 \textsuperscript{14}C-aminoacids to label the polypeptides and double labeled with [\textsuperscript{3}H]fucose to observe the comparative accumulation of fucosyl glycoprotein in the plasma membrane under these conditions. Cells were prelabeled 45 min with these precursors, the culture was divided into 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 \textsuperscript{14}C-aminoacids increased slightly in the first 7.5 min, then ceased abruptly (Fig. 5B), whereas accumulation 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.

* 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-amino acid 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-amino acids because the drug almost immediately prevented further accumulation of prelabeled protein in the plasma membranes (Fig. 5B). Therefore, in the first 30 min after the administration of the drug, its effects were considerably less on the continued accumulation of fucosyl glycoprotein in the plasma membranes than on the continued addition of [3H]fucose to the 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%). 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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
lyzed directly for trichloroacetic acid-precipitable radioactivity. Other O.1-ml aliquots were ana-
lyzed for radioactivity, and in the fractions containing radioactivity, the 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, 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). Aliquots (200 µl) of fractions were analyzed for radioactivity, and in the fractions containing radioactivity, the remainder was pooled and lyophilized. Counts per min of 4,233, 8,211, 18,100, and 104,272 from filters containing material labeled 6, 10, 15, and 60 minutes, respectively, were released by pronase digestion (see legend to Fig. 6). There was no free fucose or GDP-fucose recoverable from the pronase digests of material precipitated and washed on the filters (Fig. 6). Similar results were obtained with an experiment starting with dialyzed homoge-
nates of cells (instead of acid-precipitable material) labeled 10, 15, 20, and 60 min, except free fucose and GDP were also eluted when then 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 beha-ving as described in high voltage paper electrophoresis, was probably glycoprotein and contained no free fucose or GDP- fucose. Additional evidence confirming this conclusion is pre-
sented 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 homoge-
nates 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 bound to sedimentable (Table V).
The distribution of fucose-labeled material is shown in Table V. Fucose was glycolipid. It was investigated in the present study of HeLa cells labeled for periods up to 60 min with $[^3H]$-fucose of CHCl₃-CH₃OH (2:1).

The upper phase-labeled material remaining after dialysis, when only about 2% of the total counts were found in the upper phase.

Brain gangliosides, parallel-chromatographed as stainable standards, were all found chromatographing in the plate but with none of the fucose-labeled material from the upper phase (Table VI) or fucose-labeled standards. The bulk of the fucose-labeled material in the dianalyzed upper phase was located at the origin in paper chromatography in Solvent I (the position of GDP-fucose) and also chromatographed with GDP-fucose on Sephadex G-25 and also on paper in Solvent II.

Bosmann et al. (14) reported that about 25 to 30% of the material in HeLa cells labeled for periods up to 60 min with $[^3H]$-fucose was glycolipid. It was investigated in the present study whether a significant amount of $[^3H]$-fucose-labeled glycosphingolipid was present at short labeling times. Allkotypes of the same homogenates as in the previous experiments, labeled 6, 10, 15, and 60 min with $[^3H]$-fucose were analyzed by Foch extraction. The distribution of fucose-labeled material is shown in Table VI. The upper phase after the Foch 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). In other experiments when purified membranes were so analyzed, only about 2% of the total counts were found in the upper phase.

Upper phase-labeled material remaining after dialysis, when chromatographed on thin layer silica gel, either remained at the origin (40, 58, 61, and 83%, respectively at 6, 10, 15, and 60 min samples, respectively) or was found in the plate co-chromatographing with fucose standard. Soluble intermediates in fucose metabolism, GDP-fucose, fucose 1-phosphate, and fuconic acid run as acidic substances (see below), about 89% moved to the negative pole when washed with ethanol or CHCl₃(CH₃)OH (2:1), or CHCl₃(CH₃)OH-H₂O (10:10:3) (46). There was again no difference in the amount of radioactivity remaining on the filters when washed with the same volume of CHCl₃(CH₃)OH-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 substances and 19% was slightly acidic. When this excluded material was subsequently digested with a glycohydrolase mixture which removes most the sugars from glycoprotein oligosaccharides (see below), about 80% 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 $[^3H]$-fucose-labeled material assayed by trichloroacetic acid precipitation on filters, was glycoprotein in nature, even though much was apparently soluble in CHCl₃(CH₃)OH-H₂O. The latter property bears striking similarity to the behavior to 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 $[^3H]$-fucose and assayed by trichloroacetic acid precipitation on filters is substantially glycoprotein. Further investigation of chondroitin sulfate or heparin is essential for a more complete analysis of these materials.
thermore, there seemed little macromolecular material, even in dialyzed homogenates that was other than glycoprotein.

Time Course of Fucosyl Glycopeptide Synthesis—Glycopeptides, long term labeled (24 hours) with [3H]fucose and presumably, on average, complete in the oligosaccharide moiety, have a molecular weight of around 3000 (see "Materials and Methods"). Thus they are substantially excluded from a Sephadex G-25 column whose exclusion limit is about 3000 molecular weight (29). It was of interest to determine how quickly after the addition of fucose the maximum molecular weight of the oligosaccharide chains of these glycopeptides was achieved. This information can be derived from Fig. 6. Even at 6 min of labeling 65% of the fucosyl glycopeptide was substantially in the excluded region of the G-25 column (Fig. 6A) and by 10 min of labeling 72% (Fig. 6B) was in this region; by 60 min almost all of the fucosyl glycopeptide was in the excluded region. Ten-minute labeled material in the excluded region of the G-25 column (Fractions 33 to 13) when subsequently chromatographed on Sephadex G-50 displayed a very similar profile to glycopeptides derived from plasma membranes labeled 90 min with [3H]fucose. The latter glycopeptides, since they were derived from the site of accumulation presumably were complete. The major portion of the glycopeptides distributed heterogeneously in both cases around a peak molecular weight of 2900. With increasing labeling time, however, there was a relative increase in the ratio of excluded glycopeptide to fractions of about 2400 and 1500 in molecular weight (Fractions 36 and 40, respectively, Fig. 6A cf. Fig. 6, B, C, and D). Increase in the ratio of larger to smaller oligosaccharide chain lengths with labeling time reflected either differential rates of synthesis of small and larger species of oligosaccharides or net growth in chain lengths. There was no evidence of fucose-labeled glycopeptide intermediaries smaller than 1500 in molecular weight at any of the labeling time points (Fig. 6, A to D). Since the bulk of glycopeptides obtained from glycoproteins by exhaustive pronase digestion can be expected to have few and probably not more than 2 to 3 amino acids (average amino acid molecular weight is 120) then the oligosaccharide moiety of a species of 1500 molecular weight must contribute about 1140 in molecular weight. Assuming an average hexose residue has a molecular weight of 180 then there would be about 6 sugar residues in such a species. However, because the bulk of fucosyl glycopeptide even at 6 min of labeling was not in such small species and appeared substantially complete it can be concluded that much of the oligosaccharide chains of HeLa cell glycopeptides 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 glycopeptides 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 (25-29). 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-acetylglucosaminidase, and endo-β-N-acetylglucosaminidase D) all of which are known to be active with various (including) mammalian glycoprotein heterosaccharides. In particular, endo-β-N-acetylglucosaminidase 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" (21). 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 heterogeneous 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-
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 [3H]fucose into glycoprotein was substantially less than that which would be predicted from the reduction in specific activity of the [3H]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 [3H]fucose in the GDP-fucose pool; when equilibrated with 0.4 $\mu$M [3H]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 [3H]fucose incorporation into glycoprotein as its specific activity is decreased. Whichever of these two explanations or combination thereof applies 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 to 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 (6 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 glycoprotein oligosaccharides (40, 48, 49). Such a compound if similar in properties to dolichol-glycosyl-glycosyl 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$_2$OH-H$_2$O (10:10:3), and in this respect resembles dolichol-glycosyl-hexose (46), but was also similar to the solubilization of membrane glycoproteins from mammalian erythrocytes with CHCl$_3$-CH$_2$OH (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 [3H]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 [3H]-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-$\beta$-N-acetylgalactosaminidase 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 manno-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 (20).

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 glycopeptides 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 labeling time would seem to support a model where membrane components are added sequentially and 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 noglycosylated 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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REFERENCES

36. Hughes, R. C., and Jeanloz, R. W. (1964) Biochemistry 3, 1535-1543
Synthesis and assembly of HeLa cell plasma membrane glycoproteins and proteins.

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