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# Biosynthesis of Membrane Glycoproteins in Rat Hepatoma Tissue Culture Cells

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The early steps in the biosynthesis of glycoproteins associated with the plasma membranes of rat hepatoma tissue culture cells has been analyzed. By measuring the effect of tunicamycin on the incorporation of [$^3$H] mannose and [$^3$H] fucose into cell glycoproteins, it was determined that an interval of about 1 h was required to transfer the glycoprotein from the site of mannosylation to the site of fucosylation. This result was corroborated by an analysis of the time required for the appearance of either mannose or fucose-labeled glycoproteins at the cell surface. The separation of membrane glycoproteins by a two-dimensional gel system allowed the visualization of the modifications leading to both size and charge heterogeneity of these proteins. By following the changes in electrophoretic mobility introduced into membrane glycoproteins during a chase period after a pulse labeling, the time course of these molecular alterations could be estimated. Several glycoproteins have apparently higher rates of synthesis than the bulk of membrane-associated glycoproteins. Most of these glycoproteins were released within 2 h after biosynthesis from the intracellular membrane fraction and appear after 3 h in the medium. In addition to the glycoproteins that contain both mannose and fucose and that show a high degree of charge heterogeneity, there are other membrane-bound species that are not noticeably modified by the incorporation of fucose or sialic acids. These glycoproteins could represent constituents limited to the internal membrane system of the HTC cell.

**Key words: membrane glycoproteins, posttranslational modifications, intracellular transport, secretion**

The cellular membranes of rat hepatoma cells contain a complex mixture of glycoproteins the members of which are characterized by heterogeneity in different degrees in both size and charge [1]. This heterogeneity is mainly due to a variable extent of glycosylation in which neutral carbohydrates contribute to the size, and sialic acids to the charge, heterogeneity. The genesis of the heterogeneity in size and charge can be explained either by irregularities during the addition of monosaccharides or by subsequent partial modification of fully glycosylated molecules by cellular glycosidases. In order to understand the general pathway in the biosynthesis of individual membrane glycoproteins and the whole complex population of glycoproteins in the membrane, knowledge about the kinetics of the transfer of newly synthesized glycoproteins through the different internal membrane systems and the chemical nature of the modification occurring during this process is required. Numerous reports describing different aspects of the biosynthesis

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of glycoproteins in vertebrate cells confirm that two main features are generally applicable to the process: 1) The initial glycosylation is started by the en block transfer of a lipid-linked, neutral oligosaccharide to an asparagine residue of the nascent polypeptide chain at the rough endoplasmic reticulum [2]. This type of glycosylation can be inhibited by tunicamycin [3–5]. These first-added glycan units are enzymatically trimmed immediately after the transfer [6–10]. 2) The following stepwise glycosylation is locally and temporally separated from the above-mentioned en bloc glycosylation and is normally confined to the smooth endoplasmic reticulum and Golgi [11–14]. With these findings as a base, incorporation experiments with [$^3$H] mannose and [$^3$H] fucose were designed and are presented here; without the need of performing any complicated cell fractionation, they allow a measurement of the transition time of membrane glycoproteins through the intracellular membrane system of HTC cells. The ongoing processing of the carbohydrate structures was monitored by analysis of the glycoproteins from different stages of biosynthesis by two-dimensional gel electrophoretic techniques.

## MATERIALS AND METHODS

### Materials

Tunicamycin was a generous gift of Dr. R. L. Hamill, Lilly Research Laboratories, Indianapolis. D-[U-$^{14}$C] glucosamine (254 mCi/mmole) and L-[4,5-$^3$H] leucine (114 Ci/mmole) were purchased from Amersham; L-[6-$^3$H] fucose (16 Ci/mmole), L-[5,6-$^3$H]-fucose (60 Ci/mmole), and D-[2-$^3$H] mannose (2.7 Ci/mmole or 18.2 Ci/mmole) from New England Nuclear, and trypsin (tosylphenylalanyl chloromethyl ketone-treated; 256 U/mg) from Worthington. Concanavalin A-Sepharose was obtained from Pharmacia Fine Chemicals.

### Cells

A cloned cell line of hepatoma tissue culture cells (HTC cells) was grown in monolayer culture under an atmosphere of 95% and 5% $CO_2$ in Eagle's minimal essential medium containing 10% fetal calf serum.

### Labeling and Radioactivity Determination

In all experiments, the isotopes were first freed of solvents either by lyophilization or by evaporation, then redissolved in growth medium and added to the cells. Tunicamycin was dissolved in 60% dimethylsulfoxide (1 mg/ml) and aliquots were added to the cell culture medium. Control cultures were treated with same amounts of solvent only. Actual conditions used are described in the legends to the appropriate tables and figures. After labeling, the cells were washed three times with cold phosphate-buffered saline. For determination of total incorporated radioactivity the cells were immediately treated with 10% trichloroacetic acid. The scraped cells were sonicated, kept for 4 h at 4°C, and then centrifuged. The precipitate was extracted twice with 10% trichloracetic acid, ethanol, and ethanol-ether and then dissolved in 1 N NaOH. Protein was measured by the method of Lowry et al [15] and radioactivity by liquid scintillation counting.

### Trypsin Treatment

The proteolytic release of cell surface proteins accessible in situ to trypsin was carried out by incubation of labeled and washed cell monolayers with trypsin (5 μg/ml of phosphate-buffered saline; 1 ml/10 cm$^2$ monolayer) for 5 min at 25°C. The supernatant solution was collected, phenyl-methylsulfonyl fluoride (1 mM) was added, and any

cells that were released from the monolayer were removed by centrifugation for 5 min at $400g_{max}$. The resulting supernatant solution was centrifuged again for 60 min at $150{,}000g_{max}$. After addition of 100 μg of bovine serum albumin as carrier, the proteins were precipitated with 10% trichloroacetic acid and washed twice with 10% trichloroacetic acid, and then ethanol, and the radioactivity was measured.

### Isolation of Glycoproteins

Labeled cells were washed several times with phosphate-buffered saline and then disrupted by ultrasonication for 3 sec in 50 mM Tris-HCl (pH 7.6) containing 0.1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged for 60 min at $150{,}000g_{max}$. The pellet was washed by sonication in the above buffer and recentrifuged. The glycoproteins were extracted from the crude membrane fraction by 1% deoxycholate in 10 mM Tris-HCl (pH 8.0) and isolated by concanavalin A-Sepharose chromatography as outlined previously [1]. After elution with α-methyl mannoside, the glycoproteins were dialyzed against distilled water and lyophilyzed.

### Gel Electrophoresis

For electrophoretic analysis crude membrane fractions or isolated glycoproteins were solubilized by boiling for 2 min in 50 mM Tris-HCl, pH 6.8, containing 1% sodium dodecyl sulfate and 5% 2-mercaptoethanol. Aliquots containing between 50,000 and 200,000 acid-insoluble counts per minute in 20–30 μl were subjected to two-dimensional gel electrophoresis according to the procedure of Ames and Nikaido [16] with minor modifications as described previously [1]. The radioactive pattern was visualized by fluorography [17], which required an exposure time of 1–2 months.

## RESULTS

### Time Required for Synthesis of Membrane Glycoproteins and Insertion Into the Plasma Membrane

The core part of the oligosaccharide, after transfer from pyrophosphoryldolichol to the nascent polypeptide chain of a future membrane glycoprotein, will become in subsequent stages the accepting substrate for glycosylation reactions, leading to the formation of the complex type of a N-glycan unit [7, 10]. The addition of L-fucose and sialic acids is part of the final transfer reactions occurring during the biosynthesis of a glycoprotein [10, 11]. When cells are treated with tunicamycin, the synthesis of the substrate oligosaccharide pyrophosphoryldolichol is inhibited [3, 18] and, therefore, no initial oligosaccharide transfer occurs. The consequence of this inhibition is that the incorporation of the more distal monosaccharides into the carbohydrate structure ceases or considerably drops. By determining the inhibition of fucose incorporation at different times after pretreatment of the cells with tunicamycin, a quite valid estimate could be obtained for the minimal time required to transport newly synthesized membrane glycoproteins from the rough endoplasmic reticulum to Golgi, the locations of fucosylation [11]. The value determined in this way is presumed to represent the actual transfer time in cells not treated with the drug, if it is assumed that the antibiotic exclusively affects initial glycosylation and does not alter other steps in biosynthesis of the glycoprotein. In fact, when the influence of tunicamycin on the biosynthesis of secretory and membrane glycoproteins was studied in other systems, in some cases no significant alterations in the kinetics of membrane transport and secretion were observed [19, 20], but in other cases it was found that secretion was significantly reduced [21].

In order to apply the protocol outlined above to HTC cells, the effect of tunicamycin on total glycoprotein synthesis had first to be assessed. Table I shows that a pretreatment of the cells with 2 μg tunicamycin per milliliter for 6 h results in maximal inhibition. The incorporation of glucosamine and mannose is reduced 94%, that of fucose 88%, and leucine 22%. Further consequences of tunicamycin treatment are changes in cell shape and complete arrest in cell growth; similar reactions have been reported also for other cells [20]. Using a tunicamycin concentration of 2 μg/ml and different preincubation times, the inhibitory effect of the antibiotic on both mannose and fucose incorporation was analyzed. At this point it is important to mention that in HTC cells only a minor portion (< 5%) of the total synthesized glycoproteins represent secretory (or soluble) glycoproteins [22]. Therefore, in the following incorporation studies, the total glycoprotein fraction is considered as membrane glycoproteins. As illustrated in Figure 1, the inhibitory effect of tunicamycin on the incorporation of [$^3$H] mannose is manifested immediately after addition of the drug to the cells and reaches a maximum after 4 h. The inhibition of [$^3$H] fucose incorporation is, however, delayed for 1 h; after that it declines with the same rate as the mannose incorporation. This parallel behavior suggests a substrate-product relationship and the time difference between the onset of inhibition of mannose and fucose incorporation can be interpreted as the time necessary for precursor membrane glycoproteins to reach the location of fucosylation. Protein synthesis itself is inhibited to only a very minor extent.

In order to prove that the measured transfer times of precursor glycoproteins from rough endoplasmic reticulum to the site of fucosylation is not significantly influenced by tunicamycin, the following experiment was performed:

To HTC cells either [$^3$H] mannose or [$^3$H] fucose was added, and after different time periods the cells were treated in situ with trypsin. The radioactivity recovered in the proteolytically released fragments of the surface glycoproteins was compared with the incorporation into the total membrane glycoproteins (Fig. 2A). The time required for radioactivity to appear in the fraction releasable by trypsin (trypsinate) should be equivalent to the time needed to transport a membrane glycoprotein from the site of

**TABLE I. Effect of Tunicamycin on Glycoprotein Biosynthesis**

| | Incorporation (cpm/μg protein) | | | |
|---|---|---|---|---|
| Precursor | Control | Tunicamycin, 0.2 μg/ml | Tunicamycin, 2 μg/ml | Tunicamycin, 20 μg/ml |
| [$^3$H] Leucine | 828 | 769 | 647 | 692 |
| [$^{14}$C] Glucosamine | 826 | 433 | 68 | 51 |
| [$^3$H] Mannose | 128 | 75 | 8 | 8 |
| [$^3$H] Fucose | 359 | 205 | 45 | 34 |

HTC cells were grown in 3.5-cm dishes to half confluency. For each measurement two dishes were prepared identically. The medium (1 ml) was changed, and tunicamycin was added, yielding a final concentration as indicated. After 6 h incubation, 20-μl aliquots containing either 5 μCi [$^3$H] leucine, 4 μCi [$^{14}$C] glucosamine, 10 μCi [$^3$H] mannose (2.7 Ci/mmole), or 10 μCi [$^3$H] fucose (16 Ci/mmole) were added. The cells were incubated an additional 24 h. Then the specific incorporation into total cell protein was determined. The values represent the mean of the two identical dishes, each measurement again performed in duplicate. The amounts of radioactivity in the trichloroacetic acid-soluble fractions with [$^3$H] leucine, [$^{14}$C]-glucosamine, and [$^3$H] fucose were not affected at all by tunicamycin treatment; those with [$^3$H]-mannose were reduced up to 40% (20 μg tunicamycin/ml).

glycosylation to the plasma membrane. Previously it had been shown that the proteolytic release of surface glycoproteins from HTC cells is very reproducible and that these tryptic fragments are representative of the whole plasma membrane [23].

As illustrated in Figure 2A, the incorporation of [$^3$H] fucose into total cell protein is linear for 24 h, while that of [$^3$H] mannose is linear for only the first 6 h. The decreased incorporation after 6 h is probably due to depletion of radioactive precursor. [$^3$H] Mannose-labeled glycoproteins start to appear in the plasma membrane after 2.5 h, and 0.5 h later a temporary maximal ratio of trypsin-accessible radioactivity to total cell radioactivity is reached. The reason that the portion of mannose-labeled glycoproteins in the plasma membrane that is accessible to trypsin seems steadily to increase after 5 h is probably the lack of production of radioactive precursors. [$^3$H] Fucose-containing glycoproteins are already present in the plasma membrane 40 min after addition of the label to the cells. The maximal ratio of incorporated fucose accessible to trypsin to total cell fucose is, however, finally reached after 2 h. The result from the experiment in Figure 2A can be summarized as follows. Mannosylation occurs within a short interval of time (about 30 min) and the transport of the products to the plasma membrane requires about 3 h. Fucosylation of membrane glycoproteins, on the other hand, is spread over 1.5 h, and the insertion into the plasma membrane is maximal after 2 h. The difference of these determined transfer times (which equals the transfer time from rough endoplasmic reticulum to the site of fucosylation– spans a period of about 1 h and corroborates the previous value obtained in the tunicamycin experiment (Fig. 1).

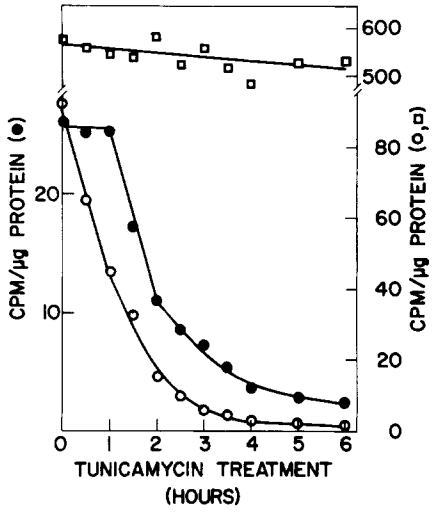



Fig. 1. Inhibition of mannose and fucose incorporation by tunicamycin. Confluent monolayers of HTC cells in 3.5-cm dishes were pretreated with 1 ml of growth medium containing 2 µg tunicamycin. Thirty minutes before termination of the incubation, 20-µl aliquots containing either 50 µCi [$^3$H]-leucine (□), 70 µCi [$^3$H] mannose (18.4 Ci/mmole) (○), or 70 µCi [$^3$H] fucose (13.12 Ci/mmole) (●) were added to the cells. For each time point, the specific radioactivities of two identical treated cultures were determined. The values shown represent the means of these two measurements. Control cultures, which were preincubated for 5.5 h in medium containing 2 µl of the solvent, dimethylsulfoxide, reached the same specific activity as those treated with the solvent only during the labeling period (value for zero tunicamycin). The amount of radioactivity in the trichloroacetic acid-soluble fraction for [$^3$H] leucine and [$^3$H] fucose was the same throughout the experiment; in the case of [$^3$H] mannose it was reduced to 20% after 6 h tunicamycin treatment.

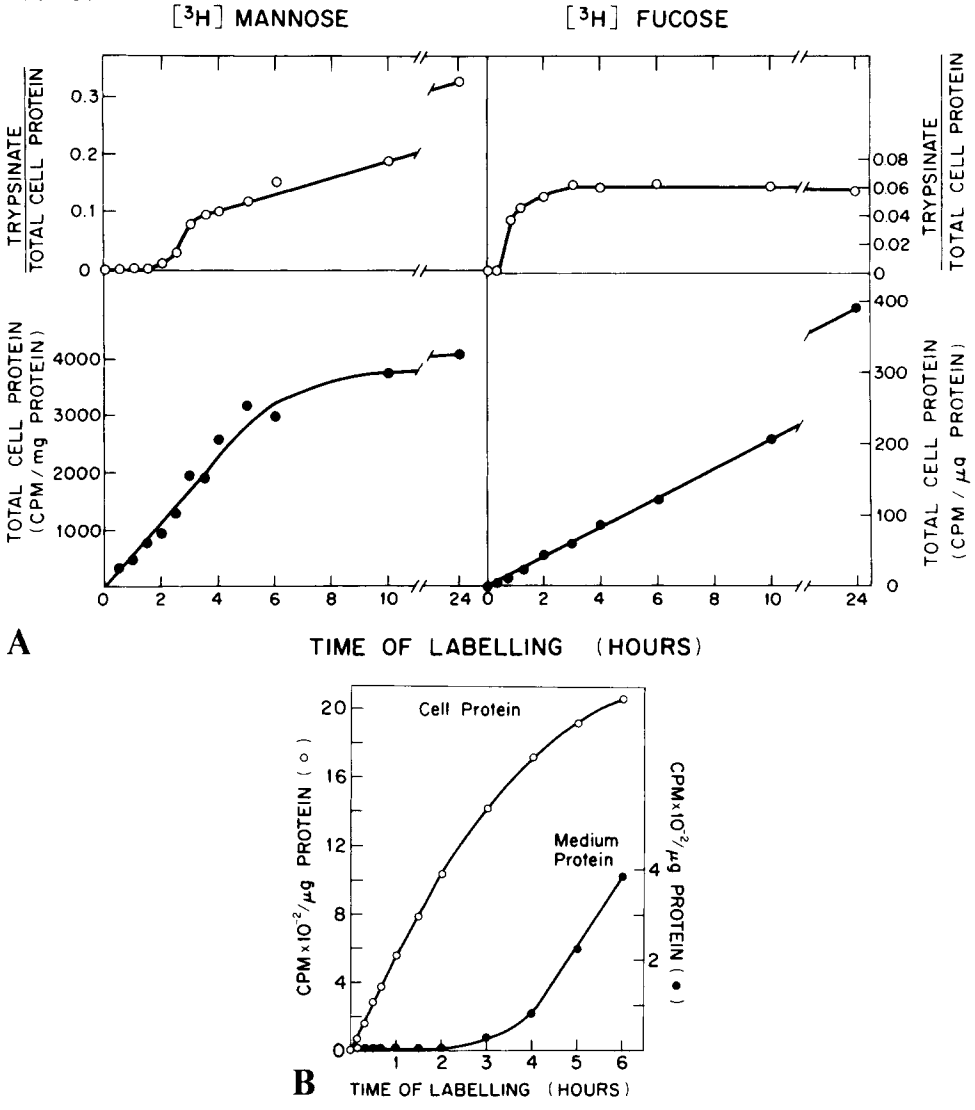



Fig. 2. Appearance of newly synthesized glycoproteins in the plasma membrane (A) and in the medium (B). A: For each determination, 4 dishes (3.5 cm) with confluent monolayers of HTC cells were incubated in 1 ml of fresh medium containing either 12 μCi [$^3$H] mannose (2.7 Ci/mmole) or 5 μCi [$^3$H] fucose (16 Ci/mmole). All incubations, except that done for 24 h, were started in such a way that they were finished at the same time. Two cultures, each identically treated, were digested in situ with trypsin. The specific incorporation and the radioactivity in the proteolytically released peptides (= trypsinate) were determined and expressed as the mean of the two measurements. B: Confluent monolayers of HTC cells in 3.5-cm dishes were incubated with 0.5 ml of fresh medium containing 80 μCi [$^3$H] mannose (18.4 Ci/mmole).

### Visualization of the Processing of Membrane Glycoproteins by Two-Dimensional Gel Electrophoresis

According to present knowledge about the biosynthesis of glycoproteins, the following behavior of the newly synthesized glycoproteins in a two-dimensional electrophoretic separation based on both charge and size can be predicted: The forms immediately after the initial glycosylation should appear as individual nonheterogenous spots.

When the maturing glycoproteins undergo further modification such as addition of neutral monosaccharides and sialic acids to the N- and O-glycan units, the glycoproteins should resolve into series of spots, indicating that the glycoprotein has acquired heterogeneity in both size and charge. The extent of this charge and size heterogeneity should also increase with time. In Figure 3 the two extreme possibilities for membrane-associated glycoproteins of HTC cells are illustrated: Those labeled within 30 min, and those labeled after 24 h, with either [$^3$H] mannose or [$^3$H] fucose. In fact, the glycoproteins containing [$^3$H] mannose, which were synthesized during 30 min and which presumably represent early precursor forms, are almost completely lacking heterogeneity. Only three noteworthy charge heterogeneous spots can be detected. After a labeling time of 24 h, however, many of the mannose-containing glycoproteins resolve into spot series showing heterogeneity in either the size or charge dimension or both. The overall pattern closely resembles that of the [$^3$H] fucose-containing glycoproteins. But there are various mannose-labeled glycoproteins that lack a corresponding spot in the pattern of the fucose-labeled glycoproteins. Some of these glycoproteins are devoid of any charge heterogeneity and throughout the labeling time remain single, well-defined spots.

A comparison of the patterns of the glycoproteins labeled with [$^3$H] mannose for 30 min and after 24 h reveals that various quite intense spots are lost (indicated by arrow heads in Fig. 3); concurrent with this disappearance from the cells, corresponding glycoprotein spot series appear in the medium, the most basic forms of which have the same molecular weight and isoelectric point as the lost mannose-labeled spots in the membrane pattern. Such assignments of a precursor glycoprotein in the cell to a glycoprotein shed or secreted into the medium was not possible however, for all secreted glycoproteins. The reason for this lies in the complex nature of the glycoprotein pattern analyzed here. That is, processing of glycoproteins by generating more acidic and higher molecular forms can be followed by the technique employed here only when precursor molecules do not undergo proteolytic size alterations. The release of such newly synthesized mannose-containing glycoproteins into the culture medium began between 2.5 and 3 h after addition of the label to the cells (Fig. 2B). This was exactly the same time that the first glycoproteins were inserted into the plasma membrane and became available to trypsin digestion (Fig. 2A).

The turnover of membrane units, as postulated in recent publications [24–26], would imply that membrane glycoproteins were also synthesized as a unit. The analysis of the [$^3$H] fucose-containing glycoproteins synthesized within 30 min yields a different pattern, however, from those after a labeling time of 24 h (Fig. 3). By comparing the relative labeling intensities of different resolved components, it appears that several fucoproteins have a higher ratio of synthesis than others. Some of these faster-synthesized glycoproteins can be found in reasonable amounts in the culture medium (marked with arrowheads in Fig. 3). This release probably explains why these glycoprotein spots are almost completely absent in the resolved pattern from long-term (24-h) labeled cells in which the accumulated "stable" membrane glycoproteins predominate. Only one of the more rapidly synthesized glycoproteins remains membrane-bound after 24 h of labeling; in addition, no corresponding form of it can be found in the medium. This glycoprotein has an apparent molecular weight of 45,000 and an isoelectric point of 5–5.5 and was recently identified as a plasma membrane glycoprotein assessible to surface labeling by the galactose oxidase and NaB[$^3$H]$H_4$ reduction method [23].

In order to demonstrate more clearly the extent of modification of some of the major mannose-labeled glycoproteins, a pulse-chase experiment was performed. Monolayers of HTC cells were labeled for 30 min with [$^3$H] mannose and then were grown in

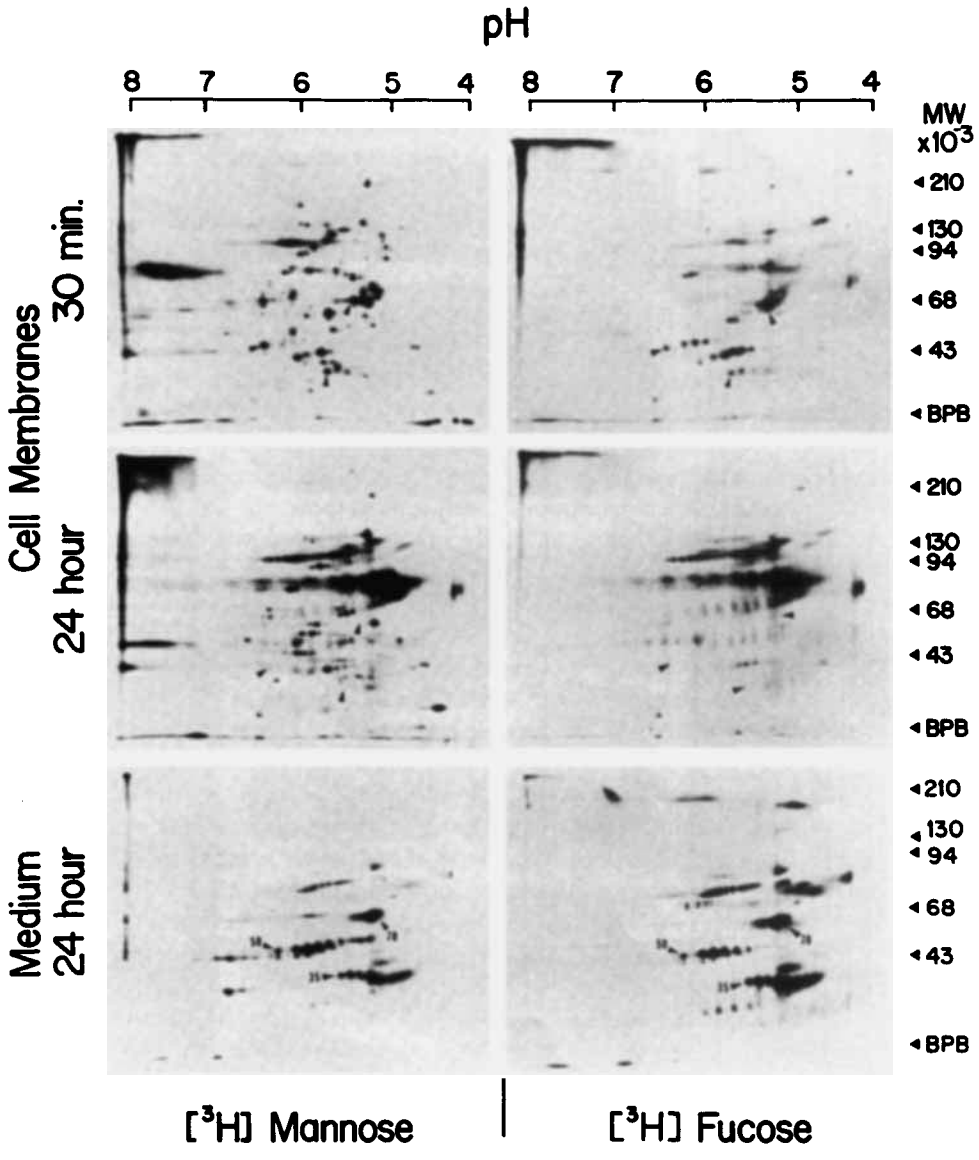
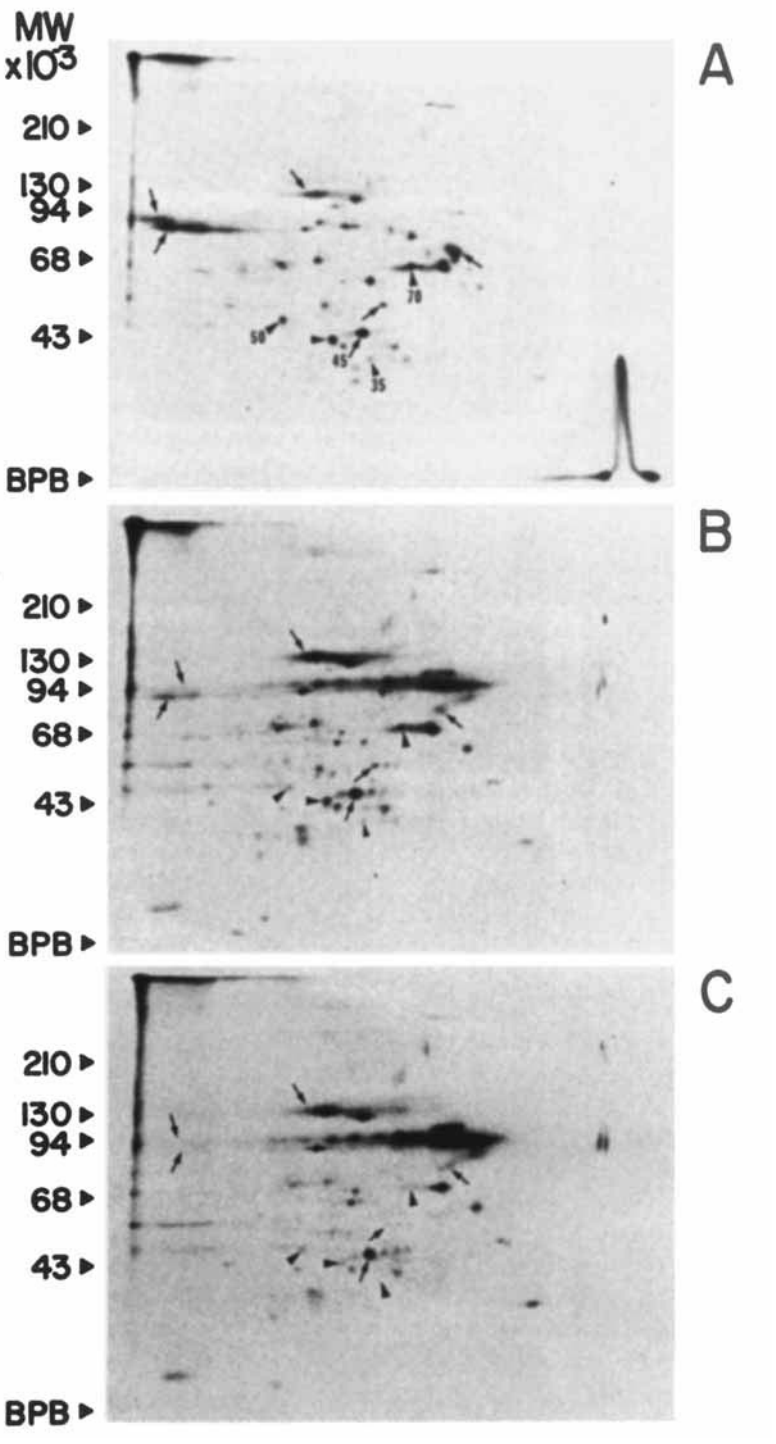



Fig. 3. Two-dimensional separation of glycoproteins labeled with either [$^3$H] mannose or [$^3$H] fucose. Confluent monolayers of HTC cells in 3.5-cm dishes were incubated in 0.5 ml of medium containing 500 $\mu$Ci mannose (18.2 Ci/mmole) or 500 $\mu$Ci [$^3$H] fucose (60 Ci/mmole). After 30 minutes or 24 h, the medium was removed, the cells were washed, and the membrane fractions were prepared. The fucose-labeled glycoproteins present in the culture medium after 24 h incubation were isolated by concanavalin A-Sepharose chromatography. Aliquots of the membrane fractions or the isolated glycoproteins containing 200,000 acid-insoluble cpm (except for membranes after 30 minutes with [$^3$H] fucose, 50,000 cpm) were analyzed by two-dimensional gel electrophoresis. The fluorographs after an exposure for 1 month (2 months for 30 minutes [$^3$H] fucose membranes) are shown. Arrowheads indicate glycoproteins that are first associated with the cellular membranes and then are released as such into the medium. The numbers mark three forms of released glycoprotein (gp 35, gp 50, and gp 70) (see also Fig. 4 and Discussion). BPB, bromphenol blue.

isotope-free medium for 1 h and 2 h. The membrane glycoproteins then were analyzed by two-dimensional gel electrophoresis and compared to the pulse-labeled glycoproteins (Fig. 4). Within the 2-h chase period, the specific radioactivity dropped about 50%. This loss of protein-bound mannose is in agreement with the findings of Robbins et al [7] and Kornfeld and his associates [9, 27, 28] that two-thirds of mannose residues in the initial oligosaccharide structure that is transferred en bloc to the glycoprotein is enzymatically removed. The reduction in specific activity was the reason why not as much radioactivity could be used for gel analysis as in Figure 3; otherwise difficulties in separation due to overloading of the gels with protein would have occurred. The experiment depicted in Figure 4 illustrates again the three different features in glycoprotein synthesis: The first is the increasing degree of charge heterogeneity during which more acidic members in a series appear, generally originating from a single charged spot (marked with arrows in Fig. 4). The two glycoproteins with apparent molecular weights of about 90,000 show the most dramatic shifts in isoelectric points, starting at pI 8 and ending at pI 5. A further noticeable feature is the appearance of two very acid glycoproteins which are located at the acid end of the first dimension and migrate with apparent molecular weight of 85,000 and 170,000. The second feature of glycoprotein synthesis is the relatively rapid disappearance of precursor forms (marked with arrowheads in Fig. 4). The third feature is the constant presence of several glycoproteins that seem to change neither in size or charge. In addition, there appears on the pulse-labeled pattern a very prominent spot that migrates as a very acid and small-molecular-weight compound (arc form in lower right corner; the shape is due to the accumulation of sodium dodecyl sulfate at the acid end of the first dimension). After only 1 h of culture in the absence of labeled mannose, this spot is already missing. When the membrane fraction of cells labeled for 30 min with [$^3$H] mannose was extracted with chloroform-methanol-water (2:1:0.1, followed by 1:1.03) [29], the spot was absent, indicating the lipid nature of this compound.

That, in fact, the label incorporated in the experiment shown in Figure 4 is confined to glycoproteins was shown by the following analysis. The total membrane fraction of HTC cells, labeled for 30 min with [$^3$H] mannose, was extracted with deoxycholate. The soluble fraction was chromatographed over concanavalin A-Sepharose, which should bind mannose-containing glycoproteins, and the bound material was analyzed by two-dimensional gel electrophoresis (Fig. 5). A glycoprotein pattern similar to that in Figure 4 was obtained. Only the proteins with molecular weight of 90,000 and above are present in relatively lower amounts. On the gel there are a few spots recognizable which correspond to the glycoproteins characterized by their persistent low-charge heterogeneity and lack of detectable fucose incorporation (in Fig. 5 indicated by arrows). On the gel in Figure 5 the strongly labeled spot in the lower right corner is present again. Because of the short lifetime and the chemical properties of this material, we tentatively conclude that this labeled compound represents dolichol-linked mannose residues.

Lectin chromatography allowed the purification of charge-heterogeneous species of glycoproteins which then could be used to determine whether the appearance of additional acidic forms in the different species (see Fig. 4) is due to increasing degrees of sialylation. For this reason, membrane glycoproteins from cells labeled with [$^3$H] mannose for 2 h were isolated and digested with neuraminidase (Vibrio cholerae, 100 units/100 $\mu$g glycoproteins) for 24 h at 37°C (for details see Baumann and Doyle [23]). The two-dimensional gel revealed that the acquired heterogeneity is partially reversed (data not shown). That the charge heterogeneity is not completely eliminated by the enzyme treatment is due most likely to the failure of the neuraminidase to remove all protein-bound sialic acid residues [23].

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## DISCUSSION

Because of its ability to specifically inhibit initial glycosylation of nascent glycoproteins [5], tunicamycin also has proved to be a useful drug for analyzing the kinetics of transfer of newly synthesized glycoproteins through the intracellular membranes of HTC cells. The time required to transfer a glycoprotein from the site of mannosylation (rough endoplasmic reticulum) to the site of fucosylation (probably Golgi apparatus

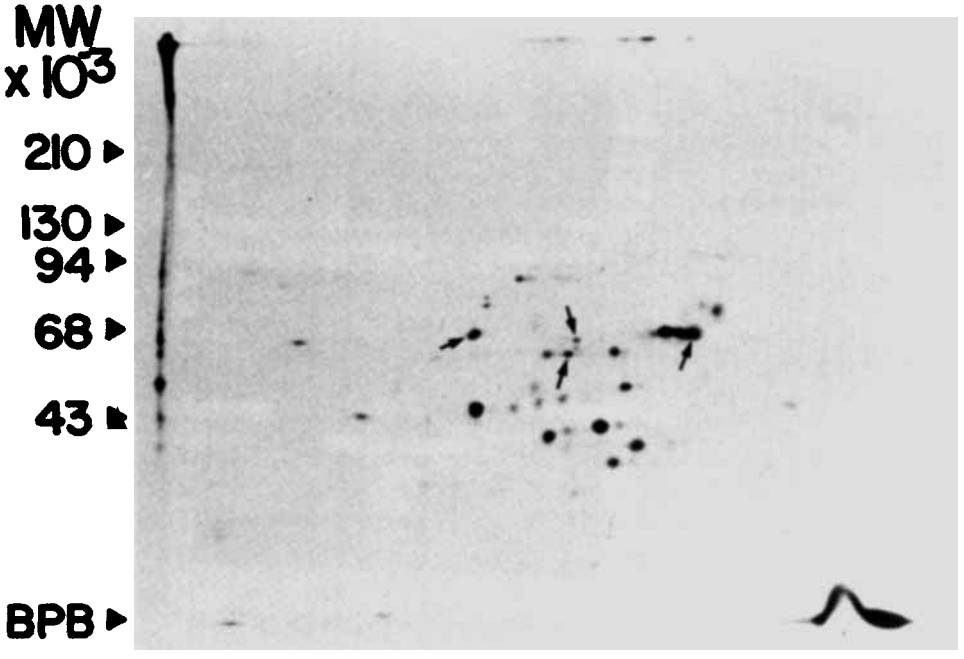


Fig. 5. Two-dimensional separation of mannose-labeled components isolated by concanavalin A-Sepharose chromatography. A confluent monolayer of HTC cells in a 75-cm$^2$ flask was incubated in 5 ml of medium containing 500 $\mu$Ci [$^3$H] mannose (18.2 Ci/mmole) for 30 minutes at 37°C. The membranes of the labeled cells were isolated and extracted with deoxycholate. The solubilized fraction was chromatographed over a column of concanavalin A-Sepharose. An aliquot of the material eluted with $\alpha$-methyl mannoside and containing 80,000 acid-insoluble cpm was subjected to two-dimensional separation. The fluorograph after an exposure of 1 month is shown. Arrows indicate the position of spots corresponding to glycoproteins, which will not develop a high degree of charge heterogeneity and which will not have detectable amounts of incorporated fucose. BPB, bromphenol blue.

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Fig. 4. Pulse-chase labeling of membranes with [$^3$H] mannose. Three dishes (3.5-cm) with confluent monolayers of HTC cells were incubated in 0.5 ml of medium with 500 $\mu$Ci [$^3$H] mannose (18.2 Ci/mmole) for 30 minutes at 37°C. After labeling, the cells were washed three times with growth medium containing 5 mM D-mannose. Two monolayers were cultured in 3 ml of medium with 5 mM unlabeled mannose for an additional 1 and 2 h, respectively. Crude membrane fractions from the cells immediately after pulse labeling (A), after 1 h chase (B) and after 2 h chase (C) were prepared. Aliquots each containing 100,000 acid-insoluble cpm were used for two-dimensional gel separation. The fluorographs after an exposure for 1 month are shown. Arrows indicate glycoproteins, which undergo an increase in charge heterogeneity during the chase period that is manifested by the appearance of additional acidic spots. Arrowheads point to glycoprotein forms which are lost during the chase period. The numbers in panel A mark the glycoproteins which have apparently higher rate synthesis than most of the bulk of the membrane glycoproteins and which have been chosen for Discussion. BPB, bromphenol blue.

[11] is about 1 h (Fig. 1). This value is in agreement with the results of Robbins et al [7, 30] and Kornfeld et al [9]. They found that processing of the initial oligosaccharide structures attached to the membrane glycoprotein of vesicular stomatis virus requires 30–60 minutes for it to become an accepting substrate for further glycosylation.
The experiments describing the effects of tunicamycin on HTC cells (Table I and Fig. 1) show an additional, quite surprising result. The incorporation of fucose is inhibited almost to the same extent as mannose. This would suggest that in membrane glycoproteins of HTC cells only a few fucose residues are attached to O-glycan structures because the biosynthesis of such structures is not supposed to be affected by tunicamycin. However, a $\beta$-elimination reaction (0.1 N NaOH, 0.1 $NaBH_4$ for 24 h at 23°C) carried out on glycoproteins isolated by concanavalin A-Sepharose from [$^3$H] fucose-labeled HTC cells depleted almost 50% of the protein-bound label (Baumann, unpublished results). Whether such a chemical treatment also removes some N-glycan-bound fucose residues cannot be answered. But the possibility cannot be ruled out that the proper formation of fucose-containing O-glycan units is directly inhibited by tunicamycin or is reduced when no N-glycan groups are attached to the precursor glycoprotein.

Looking strictly at plasma membrane glycoproteins, once the glycoproteins have reached the site of fucosylation they appear within 40 min to 2 h on the cell surface. The average time from the initial mannosylation to the insertion of these glycoproteins into the plasma membrane is about 2.5–3 h. This means that the carbohydrate modification of glycoproteins by enzymes attributed to the Golgi apparatus, such as those involved in fucosylation and sialylation [11–14], should begin at the earliest after 1 h. But according to the analysis by two-dimensional gel electrophoresis, some sialylation of various membrane-associated glycoproteins, as indicated by increasing charge heterogeneity, starts to take place within the first 30 min after synthesis (see Figs. 3–5). Apparently the modification of new glycoproteins by addition of sialic acid residues occurs somewhat earlier than fucosylation.

Several glycoprotein species associated with the membrane fraction are found to be very prominently labeled after a short incubation of HTC cells with either [$^3$H] mannose or [$^3$H] fucose. They disappear, however, within 1–2 h after the beginning of biosynthesis from the membrane fraction and can be recovered at a later stage in the medium. This relatively rapid loss is not directly due to the release of these proteins into the extracellular milieu because they are detectable first in the medium after roughly one additional hour, or 3 h after the beginning of biosynthesis (Fig. 2B). Thus, at a certain stage in their biosynthesis (after 1–2 h) these presumptive medium glycoproteins, still intracellularly located, lose their interactions with the membrane. They behave now during the cell fractionation procedure as soluble glycoproteins which were presumably released from cisternae of the microsomal membranes. This change in solubility behavior presumably takes place in the cisternal space of the Golgi and Golgi vesicles. But up until this stage, essentially all glycoproteins were transported at the same rate through the intracellular membrane system from the site of mannosylation to that of fucosylation, because no heterogeneity in the transfer time was found for any of the total cell glycoproteins (see Fig. 1). Further, both the insertion of new glycoproteins into the plasma membrane and the release of glycoproteins into the medium occur at the same time.

When HTC cells were labeled with either [$^3$H] mannose or [$^3$H] fucose for a longer period of time (24 h) and the membrane fraction was analyzed, the spots corresponding to the released glycoproteins were barely visible (Fig. 3). This suggests that these glycoproteins represent only a very minor portion of the total glycoprotein population of HTC cells.

The question arises whether these glycoproteins are authentic intrinsic membrane glycoproteins, as membrane-adsorbed glycoproteins like fibronectin, or are rapidly shed or simple secretory glycoproteins. We will limit the discussion to four prominently labeled glycoproteins, all of which exhibit a higher synthetic rate than the rest of the sugar-labeled glycoproteins: These are the glycoproteins with apparent molecular weights/isoelectric points of 70,000/5.2 (gp 70), 50,000/5.5–6.3 (gp 50), 45,000/5.2–5.5 (gp 45), and 35,000/5.2–5.5 (gp 35) (see Figs. 3 and 4). In all four cases the glycoproteins are detectable at the early stage of biogenesis (30 min of [$^3$H] mannose) as a single prominent spot. But even at the early stage of biogenesis, some minor spots appear on the acidic side of the major spot (see Fig. 3). In the pulse-chase experiment depicted in Figure 4, gp 70, 50, and 35 disappear from the membrane fraction within 2 h; glycoprotein gp 45 is, however, still present at this time and is not altered in amount. All members in the charge series of these four glycoproteins are also labeled by fucose and are detectable in the crude membrane fraction (see Fig. 3). For gp 70, 50, and 35 the same glycoproteins can be found in the medium, but for gp 45, no corresponding form is relased into the medium. When HTC cells were labeled in situ on the surface by galactose oxidase and NaB[$^3$H]$H_4$, only gp 45 was radioactive [23]. It cannot be determined, however, whether the other three proteins are not present at the cell surface at all or whether they are present but in amounts below the detection limit of the methods used to search for them. Recently, Ivarie and O'Farrell [31] reported that after dexamethasone treatment of HTC cells new glycoproteins with apparent molecular weights of 50,000 (belt I) were synthesized. The higher-molecular-weight glycoprotein (belt I) was apparently inserted into the plasma membrane, since it could be radioiodinated by lactoperoxidase treatment of intact cells. When a similar hormone experiment was carried out in which [$^{35}$S] methionine as well as [$^3$H] fucose was used for labeling, there was indeed an enhanced appearance (synthesis) of glycoproteins (belt I). And based on the behavior on the two-dimensional gel, the inducible glycoprotein was identical to the glycoprotein gp 50 discussed above (Baumann, unpublished results). Therefore, glycoprotein gp 50 apparently has some interaction with the plasma membrane, at least in hormone-treated cells, although the analysis shown in Figures 3 and 4 suggests that this glycoprotein is secretory in nature. In summary, there are glycoproteins in HTC cells, like gp 70, 50, and 35, which show higher rate of synthesis than the bulk of membrane glycoproteins. These glycoproteins are initially membrane-associated. But because they can be found in the medium, a release via secretion or shedding, rather than a rapid rate of degradation, is indicated for these glycoproteins. Glycoprotein gp 45 differs from the other three secretory glycoproteins in its behavior. Although it is synthesized in relative high rates, it is neither released as such into the medium nor accumulated at the surfaces. That means that gp 45 is transformed into another, not recognizable form or is degraded rapidly at a later stage (after 3 h).

[$^3$H] Mannose-labeled glycoproteins that are associated with the membrane yield on two-dimensional gels a pattern containing several spots for which no corresponding spots on a fucose-labeled pattern can be found. These glycoproteins are single-charged species or possess a low degree of charge heterogeneity. Analysis of the pulse-chase experiments (Fig. 4) reveals that they are not degraded within a short period of time. But neither are they detectable on the cell surface by labeling with lactoperoxidase and galactose oxidase [1, 23]. That they in fact represent glycoprotein is demonstrated by their ability to bind to concanavalin A-Sepharose (Fig. 4). The question arises whether these glycoproteins are limited to the internal membrane system of the HTC cells. The results obtained so far, however, do not allow an answer to this question. There are, in-

deed, glycoproteins present exclusively in the internal membrane system, such as the ribophorins responsible for the attachements of ribosomes to the rough endoplasmic reticulum [32].

In summary, this study shows that not all membrane glycoproteins behave identically during biogenesis. There are differences in the relative rates of synthesis, in the extent of modification of these proteins into charge-heterogeneous forms, and in the release into the environment of some membrane-associated glycoproteins.

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## REFERENCES

1. Baumann H, Doyle D: J Biol Chem 254:2542–2550, 1979.
2. Waechter CJ, Lennarz WJ: Ann Rev Biochem 41:95–112, 1976.
3. Takatsuki A, Kohno K, Tamura G: Agric Biol Chem 39:2089–2091, 1975.
4. Kuo SC, Lampen JO: Arch Biochem Biophys 172:574–581, 1976.
5. Stuck DK, Lennarz WJ: J Biol Chem 252:1007–1013, 1977.
6. Spiro MJ, Spiro RG, Bhoyroo VD: J Biol Chem 251:6400–6408, 1976.
7. Robbins PW, Hubbard SC, Turco SJ, Wirth DF: Cell 12:889–900, 1977.
8. Hunt LA, Etchison JR, Summer DF: Proc Natl Acad Sci USA 75:754–758, 1978.
9. Tabas I, Schlesinger S, Kornfeld S: J Biol Chem 253:716–722, 1978.
10. Kornfeld S, Li E, Tabas I: J Biol Chem 253:7771–7778, 1978.
11. Bennett G, Leblond CP, Haddad A: J Cell Biol 60:258–284, 1974.
12. Morre DJ: In Poste G, Nicolson GL (eds): "Cell Surface Reviews." Amsterdam: Elsevier North-Holland, 1977, pp 1–83.
13. Cook GMW: In Poste G, Nicolson GL (eds): "Cell Surface Reviews." Amsterdam: Elsevier North-Holland, 1977, pp 85–136.
14. De Pierre JW, Ernster L: Ann Rev Biochem 46:201–202, 1977.
15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265–275, 1951.
16. Ames FLG, Nikaido K: Biochemistry 15:616–623, 1976.
17. Bonner WM, Laskey RA: Eur J Biochem 46:83–88, 1974.
18. Tkacz JS, Lampen JO: Biochem Biophys Res Commun 65:248–257, 1975.
19. Duksin D, Bornstein P: J Biol Chem 252:955–962, 1977.
20. Olden K, Pratt RM, Yamada KM: Cell 13:461–473, 1978.
21. Hickman S, Kulczycki A, Lynch RG, Kornfeld S: J Biol Chem 252:4402–4408, 1977.
22. Doyle D, Baumann H, England B, Friedman E, Hou E, Tweto J: J Biol Chem 253:965–973, 1978.
23. Baumann H, Doyle D: J Biol Chem 254:3935–3946, 1979.
24. Tweto J, Doyle D: J Biol Chem 251:872–882, 1976.
25. Tweto J, Doyle D: In Poste G, Nicolson GL (eds): "Cell Surface Reviews." Amsterdam: Elsevier North Holland, 1977, vol 4.
26. Baumann H, Doyle D: J Biol Chem 253:4408–4418, 1978.
27. Li E, Tabas I, Kornfeld S: J Biol Chem 253:7762–7770, 1978.
28. Tabas I, Kornfeld S: J Biol Chem 253:7779–7786, 1978.
29. Waechter CJ, Lucas JJ, Lennarz WJ: J Biol Chem 248:7570–7579, 1973.
30. Hubbard SC, Robbins PW: J Biol Chem 254:4568–4576, 1979.
31. Ivarie RD, O'Farrell PH: Cell 13:41–55, 1978.
32. Kreibich G, Czako-Graham M, Grebenau R, Mok W, Rodriguez-Boulan E, Sabatini DS: J Supramol Struct 8:279–302, 1978.