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INTERNALIZATION, INTRACELLULAR SORTING, AND RECYCLING OF CELL SURFACE SIALOGLYCOPROTEINS IN HeLa CELLS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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ABSTRACT

Cell surface sialic acid residues were specifically modified to a radiolabeled, neuraminidase-sensitive, 7-carbon analog ([^{3}H]-AcNeu7) by sequential treatment of HeLa or HTC cells with $NaBH_4$, $NaIO_4$, and $NaB[^3H]_4$. [^{3}H]-AcNeu7 is neither reutilized nor is it metabolized by intact cells. With time (approx. 30 hours) cell surface [^{3}H]-AcNeu7-glycoconjugates equilibrate with the intracellular milieu. Approximately 30% of the cell associated radioactivity is then sensitive to hydrolysis by added extracellular neuraminidase. Cells allowed to internalize cell surface [^{3}H]-AcNeu7-glycoconjugates and subsequently exposed to neuraminidase to remove surface radioactivity contain an intracellular pool with a relatively high specific radioactivity compared to the plasma membrane. Resuspension of these cells in growth medium and assay of neuraminidase-sensitive radioactivity at various times thereafter show a return of labeled glycoconjugates to the cell surface again reaching euqilibrium with approximately 30% of the cell-associated radioactivity sensitive to neuraminidase. After labeling, approximately 16 labeled sialoglycoproteins could be identified by SDS-PAGE, including 7 major bands. All of the major bands are found in the internal pool and are seen to associate first with an endocytic compartment, and are then transferred to the

lysosomes, reaching a peak in the lysosomes between 30 and 60 min. Material can then be transferred to the Golgi complex 1 to 3 h after labeling, and also, perhaps, to a post-Golgi compartment (which may be the GERL?) from which it recycles back to the cell surface. Characterization of the material associated with these various fractions by SDS-PAGE reveals that all the major bands are found in both the endocytic compartment and in lysosomes treated with NH_4Cl to impair degradation. In lysosomes from untreated cells, several bands are missing, and presumably degraded. Sorting is observed to occur at the level of internalization, during association of material with the endocytic compartment, and via specific degradation. No further sorting was observed after the material had been associated with the lysosomal compartment.

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ABBREVIATIONS

HTC	rat hepatoma tissue culture
S-EMEM	Eagle's minimum essential medium with Spinner salts
PBS	phosphate-buffered saline
PPO	2,5 diphenyloxazole
DMSO	dimethylsulfoxide
FD	fluorescein-isothiocyanate dextran
CF	cationic ferritin
TCA	trichloroacetic acid
PTA	phosphotungstic acid
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrohporesis
BPB	bromophenol blue
EGF	epidermal growth factor
GERL	Golgi-endoplasmic reticulum-lysosomes

CHAPTER I

THE FATE OF THE CELL SURFACE: AN OVERVIEW

The ability of cells to grow both *in vivo* and *in vitro* is dependent on a complex interaction of the cell with its extracellular environment. The strict cellular requirement for various exogenous molecules may be met through either their interaction at the cell surface, as is the case for neurotransmitters (Kahn, 1976; Raftery et al., 1980), insulin (Kahn et al., 1978), and thyrotropin (Rees Smith and Buckland, 1982) for example, or they may have to act intracellularly, such as transferrin or other essential metabolites (e.g., sugars, amino acids, ions, nucleosides). The above compounds enter the cell through a specific interaction at the cell surface with a receptor or transport protein, and it is through the concentration of these surface molecules or their affinity for ligand that the cell can regulate the rate of entry of these extracellular molecules, thus allowing cells to regulate their effect.

In the case of epidermal growth factor (Carpenter and Cohen, 1979), low density lipoprotein (LDL; Goldstein and Brown, 1977), or transferrin (Ward et al., 1982), the internalization of the receptor-ligand complex occurs via

a pinching off and internalization of a part of the plasma membrane in the form of a vesicle (Silverman et al., 1977). This intracellular movement of the receptor-ligand complex is referred to as receptor-mediated (adsorptive) endocytosis. This form of endocytosis allows for the specific and highly-regulated entry of various exogenous molecules into the cell. Most of the receptor-mediated endocytosis is found to occur in specialized regions of the plasma membrane referred to as coated pits (Goldstein and Brown, 1977; Goldstein et al., 1979; Carpentier, et al., 1982). Receptor-ligand complexes become concentrated in coated pits, at which point the cell surface can invaginate this pit into a coated vesicle (Heuser, 1980). Coated pits and vesicles are characterized by a bristly appearance of the intracellular side of the plasma membrane. This bristly appearance is due to the presence of a variety of proteins, mainly clathrin (Perry and Gilbert, 1979; Willingham et al., 1979; Ungewickell and Branton, 1981; Pearse and Bretscher, 1981).

Clathrin (Mol. wt. 180,000), spontaneously forms triskelions, which bind to proteinaceous core particles (Pearse, 1982) composed of a variety of different polypeptides. Proteins of molecular weight 30,000 to 36,000, which are referred to as light chains or clathrin-associated proteins (CAPs; Daiss and Roth, 1983)

are found tightly bound to clathrin upon purification. Several other groups of proteins are also present in purified coated vesicle preparations, having molecular weights of 50,000 to 55,000, including both α- and β-tubulin (Pfeffer et al., 1983), and protein(s) with a molecular weight of approximately 100,000 (Pearse, 1978; Ungewickell and Branton, 1981). Clathrin, however, still accounts for 30 to 70% of the total protein of coated vesicles (Pearse, 1983). In the presence of the CAPs, 36 triskelions form a polyhedral structure referred to as the clathrin basket, composed of 12 pentagons and 8 hexagons. Though many receptors internalized via coated vesicles are normally found, even in the absence of ligand, residing primarily in the coated pit regions of the cell surface, one major exception is the insulin receptor. Insulin receptors have been found to be distributed throughout the cell surface in fibroblasts. Upon binding of ligand they become associated within the coated pits (Pastan and Willingham, 1980). It is thought that the binding of ligand induces a conformational change in the insulin receptor, resulting in its recognition by a coated pit protein. When ligand is bound and the insulin receptor is internalized via coated pits, many of these receptor molecules are degraded. The reduction in the number of insulin receptors on the cell surface is referred to as

down regulation (Kahn and Roth, 1975; Raff, 1976). Down regulation, therefore, allows the concentration of insulin in the medium to regulate, at least in part, the level of receptors on the cell surface.

A second form of endocytosis also occurs, and is referred to as fluid-phase endocytosis. Vesicles formed by the invagination of the cell surface and its subsequent pinching off to form intracellular vesicles carry with them extracellular fluid, and thus internalize whatever materials are present in this extracellular fluid. This type of endocytosis is nonspecific, and, in fact, may be occurring, to a large extent, just as a consequence of receptor-mediated endocytosis. The extent of coupling of these two forms of endocytosis is at this time unknown.

The cell, therefore, is continually internalizing its surface as a consequence of endocytosis, and a mechanism for the replacement of this internalized surface material must be continually at work, since the area of the cell surface and the cell volume are virtually unchanging. It was believed that the internalization of the cell surface led to the degradation of surface components, which were replaced via biosynthesis. However, in 1976, Steinman and his co-workers (Steinman et al., 1976) demonstrated that the rate of degradation of surface components was slow (less than 2%/h) compared to the rate of internalization

of the cell surface, which they found to be as high as 1-2 surface equivalents/h in macrophages. The only possible explanation for such a disparity was that the internalized surface material was being reused, i.e. being recycled back to the cell surface. This observation prompted a number of laboratories to begin investigations into the recycling of internalized cell surface components.

Antibodies specific for various cell surface antigens have been used to detect internalization and recycling of the cell surface in several laboratories (Schneider et al., 1979b; Louvard, 1980; Widnell et al., 1982). The cells are exposed to the primary antibody, allowed to internalize their surface, and the reappearance of the internalized antibody-antigen complex can be detected returning to the cell surface by the use of a secondary antibody. This technique, however, cannot be relied upon to give accurate results, since antibodies had been shown to protect antigen from degradation (Schneider et al., 1979a). It is highly likely, therefore, that the use of antibodies perturbed the kinetics of the turnover of surface-derived material, and may have, in fact, caused the recycling of molecules which were normally degraded as a consequence of their internalization. Another method that has been employed to study recycling is protease treatment. Protease treatment of the plasma membrane

resulted in the abolishing of certain enzymatic and/or transport activities, which, in the presence of protein synthesis inhibitors, could be observed to return to the cell surface from, presumably, an intracellular pool(Stahl et al., 1980). Such harsh treatment of the cell surface could have resulted in non-physiological responses from the cells undergoing this protease treatment.

The electron microscope has been used to follow the uptake of fluid-phase and adsorptive endocytic markers to analyze recycling. Several studies have been published utilizing horseradish peroxidase as a fluid-phase marker which have observed a route of movement from the cell surface to the phagolysosomal compartment (Steinman et al., 1976; Willingham et al., 1983). Other studies have relied upon the electron-dense nature of metal compounds, such as cationic and anionic ferritin and native gold for visualization of cell surface components microscopically. Ferritin, when added to cells, is adsorbed nonspecifically to the cell surface. These studies have revealed that a variety of pathways are at work in a number of cell types. Van Deurs and Nilausen (1982), using cationic ferritin bound to the surface of rat fibroblasts, have visualized what they believe to be a shuttle of microvesicles (≅100 nm) between the plasma membrane and the lysosomes. Farquhar (1978) finds that less than one percent of the

cell surface in IgG-secreting murine myeloma cells is associated with lysosomes after internalization, but extensive labeling of the Golgi stacks was observed.

Other studies have yielded conflicting results concerning the involvement of the Golgi complex in the process of recycling. In some studies (Farquhar, 1978; Herzog and Miller, 1980; Thyberg and Stenseth, 1981), cationic ferritin bound to the cell surface has labeled the Golgi stacks, whereas in other studies, there is a lack of cationic ferritin labeling of the Golgi stacks (Tartakoff et al., 1981; Van Deurs et al., 1981; Van Deurs and Nilausen, 1982). Cationic ferritin has been shown to bind and unbind under various conditions, which may explain the discrepancy in the literature concerning the association of internalized cell surface material with various intracellular organelles.

Evidence has been presented which suggests that the contents of endocytic vesicles are treated differently than the vesicular membrane components. When both anionic ferritin and cationic ferritin were used as markers for both fluid-phase and receptor-mediated endocytosis, respectively, it was found by Farquhar (Farquhar, 1978) that upon internalization, anionic ferritin is associated only within the lysosomes, whereas cationic ferritin was associated not only within the lysosomes, but also within

the Golgi, condensing granules and also the GERL (Golgi-endoplasmic reticulum-lysosomes), all elements involved in the secretory process. Antibodies have been covalently labeled with gold as a marker for use during electron microscopy, however they have the same drawback outlined in Chapter I for use in the quantitation of recycling: the protection of the antigenic site by the antibody. Thus, the techniques available for the study of recycling were creating the possiblities of anomalous results, further clouding the study of recycling.

The possible movement of material from the lysosomal compartment to the cell surface was approached in a novel fashion by Muller, Steinman, and Cohn (1980), who coupled lactoperoxidase to latex beads, which could then be phagocytosed by macrophages. The beads were subsequently accumulated in the lysosomal compartment. Na[^{125}I] was then attached to the cells, and it also accumulated in the lysosomes. In the presence of lactoperoxidase, ^{125}I from Na[^{125}I] is covalently added to tyrosine residues of proteins. Incubation of the macrophages at 37^{o}C resulted in the appearance of the labeled lysosomal proteins on the cell surface. Thus, the movement of lysosomal proteins to the cell surface was established, lending credence to the possibility that material associated with the lysosomal compartment could escape and possibly recycle from this

compartment (Van Deurs and Nilausen, 1982). The lysosomal compartment may not be, therefore, the degradative dead-end it was once believed to be.

Specific cellular systems such as the insulin and insulin receptor system were used to analyze the sequence of movement of surface derived material through various intracellular organelles. Morphological studies (Golfine et al., 1977; Gordon et al., 1978; Carpentier et al., 1979; Bergeron et al., 1979) have shown internalization of insulin results in the insulin being found first in the Golgi/GERL region, followed by its progressive movement into lysosomal-like structures. Krupp and Lane (1982) found that chloroquine, which disrupts the lysosomal pH gradient and interferes with the lysosomally dependent degradation, also caused an increase in the levels of cell-associated radioactive insulin, but had no effect on the level or rate of degradation of insulin receptors. In fact, Krupp and Lane also found that the average insulin receptor is recycled approximately 200 times prior to its degradation.

It is currently believed that two or more pathways may exist for the degradation of many cellular proteins (Goldberg and Dice, 1974; Goldberg and St. John, 1976; Knowles and Ballard, 1976; Amenta and Brocher, 1981). Proteins with short half-lives ($t/2 \cong 7$-10 h) are degraded

by an ATP-dependent process which might involve the protein ubiquitin, whereas other proteins with a longer half-life seem to be degraded via a lysosomally dependent pathway (Neff et al., 1979). The insulin receptor, with a half-life of approximately 10 h (Krupp and Lane, 1982) may use the ATP-dependent, non-lysosomal pathway, whereas other proteins with longer half-lives may be degraded through the lysosomal pathway. Devreotes and Fambrough (1975) report that the half-life for the acetylcholine receptor is approximately 29 h. It was also reported (Libby et al., 1980) that the treatment of chick myotubes with chloroquine resulted in an increased number of acetylcholine receptors associated within the lysosomal compartment of the cell, indicative of what would occur if the acetylcholine receptor was degraded lysosomally, in contrast to the effects described for the insulin receptor system. The prolactin receptor in mammary gland explants has been reported by Djian et al., (1980) to be degraded in a lysosomally dependent manner.

The non-lysosomally dependent pathway of degradation could also be important in continuing maintenance of the integrity of the cell surface. Although steady state turnover seems to be a random process, proteins containing abnormal amino acids are degraded at an accelerated rate (Goldberg and Dice, 1974). An alteration in the primary

structure may confer changes in both the tertiary and quartenary structure of the protein, which in turn may allow for the recognition of this protein by the rapid, non-lysosomal method of degradation. Correlates *in vivo* may be the damage of plasma membrane proteins by surface and/or extracellular proteases, or damage may occur simply via mechanical stress encountered at the cell surface. Such a mechanism would thus allow for the rapid removal of damaged proteins, thus ensuring the good state of repair of the cell surface. Alternatively, the report reviewed above concerning the escape of lysosomal proteins and their movement to the cell surface (Muller et al., 1980) suggests a simpler method for removing damaged proteins may be in operation: conformational changes, resulting from damage at the cell surface or mistranslation of the protein, could result in proteins' being significantly more susceptible to degradation once associated with the lysosomal compartment. The rate of endocytosis being up to 2 cell surface equivalents/h would allow for the continual screening of plasma membrane proteins by passing them through the lysosomal compartment with the apparent degradation of damaged proteins occurring as needed.

The apparently large number of pathways available for internalization and degradation of plasma membrane and extracellular molecules leaves the cell with an enormous

problem of sorting out these molecules from one another as well as directing each type of molecule to its ultimate destination. Although the actual mechanism of sorting is not understood at this time, there is evidence that events at or near the surface of the cell may be of paramount importance. Lodish and co-workers (Ciechanover et al., 1983) have shown that the asialoglycoprotein receptor is internalized independently of the insulin and transferrin receptors, though at least the insulin receptors and asialoglycoprotein receptors are internalized through coated pits (at this time, it is believed that transferrin is also internalized via coated pits; see also Bleil and Bretscher, 1982). Thus, in this case, the internalization of the cell surface does not cause an internalization of a random sampling of the cell surface, but in many cases may be a specific internalization of a class of receptors or other proteins. Other recent studies, including that of Willingham et al. (1983), have shown that epidermal growth factor undergoes internalization with adenovirus particles and alpha-2-macroglobulin into the endocytic compartment and eventually into another organelle which was recently termed the receptosome.

The receptosome (Willingham and Pastan, 1980; Willingham et al., 1983), seems to be derived from coated vesicles, which bud off part of their membrane to form an

uncoated vesicle. This uncoated vesicle, the receptosome, fuses with other such vesicles, and makes its way to the Golgi complex, where it becomes associated with Golgi elements. These receptosomes do not seem to fuse with lysosomal vesicles (Willingham and Pastan, 1981), and therefore, may provide a pathway from the plasma membrane to the Golgi complex, bypassing any association with the lysosomal elements. Endosomes, also originating from internalized cell surface material, seem to fuse primarily with primary and secondary lysosomes. As was reviewed above, several recent studies have indeed shown that upon internalization, insulin is found first associated with the Golgi and/or GERL (Goldfine et al., 1977; Gordon et al, 1978; Carpentier et al., 1979; Bergeron et al., 1979) and then moves to the lysosomes. It has also been demonstrated that a variety of ligands can be separated from their receptors due to the rapid acidification which occurs in the endocytic vesicle. This acidification occurs within one minute or so of the pinching off of the coated vesicle from the cell surface (Tycko and Maxfeld, 1982; Tycko et al, 1983). A number of ligands separate from their receptor under acidic conditions (pH=5.5-6.5), among them LDL (Basu et al., 1978), asialoglycoproteins (Bridges et al., 1982), epidermal growth factor (Haigler et al., 1982), lysosomal enzymes (Gonzalez-Noriega et al.,

1980; Tietze et al., 1982), and alpha-2-macroglobulin (Maxfeld, 1982).

In the case of lysosomal enzymes, it is known that a portion of the lysosomal enzymes are secreted into the medium and recaptured (Hasilik and Neufeld, 1980), via receptor recognition of a mannose-6-phosphate on the enzyme molecule. Since it is also known that primary lysosomes originate from the GERL region of the Golgi, the above receptor-mediated recapture of lysosomal enzymes from the extracellular medium may result in the delivery of these enzymes to the Golgi complex, either directly or indirectly, for packaging into primary lysosomes. In addition, Krupp and Lane (1982) have shown that insulin and the insulin receptor follow distinctly different pathways once inside the cell, again indicating a possible separation of ligand and receptor. In the case of LDL, the ligand is delivered to the lysosomes (Anderson et al., 1982) and is, therefore, unlikely to segregate via the receptosome system. Since acidification of the endocytic vesicle does result in LDL being dissociated from its receptor (see above), there must be another force keeping LDL out of the receptosome, and thus directing it towards the lysosomes. The size of the LDL particle ($\cong$22 nm) and associated lipid bilayer coat may keep the LDL particle associated with the endosome, unlike the other ligands

described above, which may associate with receptosomes for delivery to the Golgi complex.

Interruption of the acidification of the endocytic vesicle has been shown to inhibit the movement of LDL (Anderson et al., 1982) to the lysosomes, and caused a depletion of cell surface receptors for lysosomal enzymes (Gonzalez-Noriega et al.,1980). It was suggested that the inhibition of vesicle acidification (i.e. inhibition of ligand-receptor separation) could entrap receptor-ligand complexes intracellularly. The inability of ligand to separate from its receptor may, therefore, result in the inhibition of receptor recycling (Gonzalez-Noriega et al., 1980; Anderson et al., 1982). When in the presence of monensin, which dissipates the pH gradient in endocytic vesicles and lysosomes (Pressman, 1976), fluorescent LDL was found to accumulate in the same vesicular compartment as the LDL-receptor (Anderson et al., 1980). The acidification of the endocytic vesicle was also shown to be responsible for the penetration of enveloped viruses into the cytoplasm (Marsh et al., 1982). The internalization of receptor-bound bacterial toxins (Keen et al., 1982) into acidified vesicles may allow for the direct entry of these toxins into the cytoplasm (Marnell et al., 1982). The full implications of the above acidification of the endocytic vesicle and the role it plays in the sorting of

internalized surface and surface-bound material remains to be elucidated.

n an effort to gather information on the movement of various cell surface proteins into the cell, free from the possible side-effects of antibodies, EM tracers, and/or protease use as outlined above, I have modified a labeling technique first described by Van Lenten and Ashwell (1971) which allows the incorporation of tritium into externally exposed sialic acid residues. This labeling allows cell surface sialoglycoconjugates to be followed via a number of qualitative and quantitative methods, in an effort to study their movement during endocytosis and recycling. By using such a labeling system, measurements of recycling of plasma membrane components in both HTC and HeLa cells were collected, and a route for the movement of internalized surface material through various intracellular organelles in HeLa cells was elucidated, and are presented below.

CHAPTER II

RECYCLING OF CELL SURFACE SIALOGLYCOCONJUGATES IN HTC AND HeLa CELLS

Introduction

It is well established, as outlined in Chapter I that a variety of cell surface molecules are not degraded upon internalization, but are reutilized and returned to the cell surface. The data supporting the recycling of cell surface molecules have been accumulated via the use of techniques which themselves may have contributed to material recycling by interfering with or altering the normal cellular homeostasis. Many of the studies were based on work performed using antibodies, shown to protect antigens from degradation (Schneider et al., 1979b).

To study recycling, I took advantage of a technique which specifically labels externally oriented sialic acid residues (Van Lenten and Ashwell, 1971; Gahmberg and Andersson, 1977), but by virtue of the fact that it causes only a minor alteration in the structure of cell surface sialoglycoconjugates, does not significantly perturb the physiological state of the cells. $NaIO_4$ oxidation under carefully controlled conditions (Warren, 1957; Van Lenten and Ashwell, 1971; Gahmberg and Andersson, 1977; Dodeur

and Bourrillon, 1978) cleaves the exocyclic triol of sialic acid converting it to an aldehyde, allowing tritium incorporation on subsequent reduction with $NaB[^3H]_4$. The resulting sugar from the above reaction, [^{3}H]-5-acetamido-3,5-dideoxy-L-arabino-heptulosonic acid, abbreviated AcNeu7, is the major product of the reaction. The octulosaminic form (AcNeu8) accounts for about 6% of the total reaction product. The labeled sugar can be removed with neuraminidase, though it is released at a somewhat slower rate than is sialic acid (Blumenfeld et al., 1972). Minor cell components, primarily lipid peroxides and plasmalogens are also labeled by this method (Blumenfeld et al., 1972), though this can be reduced to about 5% of the total incorporated radioactivity by pretreating the cells with NaBH (Baumann and Doyle, 1978).

The reduction of exclusively surface molecules by $NaB[^3H]_4$ has been shown using electron microscopic autoradiography of thin sections of labeled HTC cells (Baumann and Doyle, 1978) as well as by the lack of labeling of sialoglycoproteins trapped inside resealed red blood cell ghosts (Gahmberg and Andersson, 1977). Cell growth rates are also unaffected by the modification of the sialoglycoconjugates. In studies on blood plasma glycoproteins labeled in this way, the clearance rate of the modified ceruloplasm and orosomucoid from the rat

circulatory system was unchanged (Van Lenten and Ashwell, 1971). These results suggested that the above labeling protocol could provide a system of negligible perturbation to the physiological state of the cell. Evidence is presented below for the recycling of surface membrane sialoglycoconjugates between the plasma membrane and an internal pool in HTC and HeLa-S3 cells.

Materials and Methods

Chemicals

Eagle's minimum essential medium with spinner salts (S-EMEM) and L-glutamine were from GIBCO, gentamicin (Schering) from Microbiological Associates, and HEPES was from Calbiochem. Heat inactivated fetal bovine serum was obtained from GIBCO or Kansas City Biological Company. Neuraminidase from Clostridium perfringens was from Sigma, neuraminidase from influenza virus was from Calbiochem, thiobarbiturate was from Eastman Kodak. Carrier-free $NaB[^{3}H]_4$ (226.6 mCi/mmol) and [^{14}C] glucosamine (52 mCi/mmol) were from New England Nuclear. Reagents for use in SDS-PAGE were from Bio-Rad. All other chemicals were of the highest grade commercially available.

Cell Culture

HTC (Hepatoma tissue culture) cells, a gift from Dr. D. Doyle (Roswell Park Memorial Institute, Buffalo, New York) and HeLa-S3 cells (Puck et al.,1956) were grown in suspension in S-EMEM (Eagle, 1959) supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 25 micrograms/ml gentamicin, and 20 mM HEPES (pH 7.4). The concentration of $NaHCO_3$ was also reduced to 2.5 mM. Cultures were swirled at 110 cycles per min at 37° C in screw-cap Erlenmeyer flasks. Periodic tests showed the cultures to be free of mycoplasma. Cell number was monitored with a Coulter Counter, Model B (Coulter Electronics. Hialeah, FL). Cells were harvested during exponential growth (2-8 X 10^5 cells per ml).

Isolation of Plasma Membranes

Plasma membranes were isolated as was previously described (Atkinson, 1973; Pollack et al., 1981a). Membranes were resuspended in 10 mM Tris/HCl, pH 7.4, and stored at -80° C.

Assays

Protein was assayed by the fluorometric method of Avruch and Wallach(1971); 5'-Nucleotidase (E.C. 3.2.3.5) activity was also determined by the method of Avruch and

Wallach (1971) as modified (Brake et al., 1978); and β-hexosaminidase (E.C. 3.2.1.30) was by the method of Barret (1972). Sialic acid and AcNeu7 were assayed by the thiobarbituric acid method of Warren (1959) or by a modification of the fluorescence assay of Murayama et al. (1976). In the latter, the pyridoxamine:AcNeu7 ratio was increased to 50:1 and the solution was immersed in the 70° C water bath for 60 min. Assay of the AcNeu7 was routinely performed with the pyridoxamine assay to avoid inconsistencies observed when using the thiobarbiturate method.

NaB[^{3}H]$_4$-labeling of HTC and HeLa Cells

1-4 X 10^8 cells were collected in 125 ml Bellco glass centrifuge bottles and washed three times with S-EMEM. Cells were resuspended at a concentration of 5 X 10^6 cells per ml and exposed to 2 mM $NaIO_4$ for 10 min at 4° C. Then 0.02 ml of 10% glycerol per ml cell suspension was added with shaking to react any $NaIO_4$ remaining. The cells were washed twice and resuspended in a total volume of 5 ml to which 2 or 5 mCi of carrier- free NaB[^{3}H]$_4$ was added in 0.2 ml of ice-cold 0.01 N NaOH. After incubation for 10 min on ice, 1 mM $NaBH_4$ was added to reduce any remaining aldehydes on the surface. The cells were then washed exhaustively with PBS until the background radioactivity

was insignificant. This labeling procedure yielded the highest specific radioactivity with cells that had reached less than 5 X 10^5 cells/ml in their growth medium.

Gel Electrophoresis

Discontinuous SDS-PAGE was carried out utilizing a resolving gel of 9% as described by Laemmli (1970). Gels were stained with Coomassie Brilliant Blue or subjected to fluorography with 2,5-diphenyloxazole (PPO) and dimethyl sulfoxide (DMSO) as described by Bonner and Laskey (1974). Gels were dried and exposed to Kodak XAR film for 14 days at -70° C with an enhancing screen. Molecular weight standards, purchased from Bio-Rad were: myosin, 200,000; β-galactosidase, 116,500; phosphorylase a, 94,000; bovine albumin, 68,000; and ovalbumin, 43,000.

Neuraminidase Treatment of Cells

For treatment of large numbers of cells (greater than 5 X 10^7), cells were suspended at a concentration of 1 X 10^6 cells per ml in S-EMEM which was supplemented with 1 mM $CaCl_2$ and a mixture of 2 units (Warren, 1963) per ml neuraminidase from Clostridium perfringens, and 10 units per ml neuraminidase from influenza virus, pH 5.0. For small numbers of cells, 10 units per ml C. perfringens neuraminidase plus 20 units per ml of influenza virus neuraminidase were added to the same medium in a total

volume of 2-3 ml. Neither the labeling procedure nor the neuraminidase treatment significantly altered the growth rate of the cells. For cells that were not going to be resuspended (cf. Fig.1), the neuraminidase treatment was performed in PBS at pH 5.0 rather than in S-EMEM.

Acid Hydrolysis of Labeled Cells

$NaBH_4/NaIO_4/NaB[^3H]_4$-treated cells were subjected to acid hydrolysis in 0.125 N sulfuric acid for 1 h at 80° C. The hydrolysate was then neutralized with 1 N NaOH and centrifuged at 25,000 X g for 60 min in a Spinco SW56 rotor. The supernatant could then be analyzed by paper chromatography for labeled monosaccharides.

Chromatography

Paper chromatography was performed on Whatman 3 MM paper in one of the following solvent systems: (A) n-butyl-acetate/ acetic acid/water (3/2/1, v/v/v); or (B) n-butanol/n-propanol/0.1 N HCl (1/2/1, v/v/v). The chromatograms were then developed by descending chromatography for 12-15 h. After drying, 1 cm strips were cut and the material eluted overnight with 1 ml water for 2-inch wide chromatograms. Nine ml ACS scinitllation fluid was added and the samples were then counted in a Packard Tri-Carb Scintillation Counter. For larger chromatograms, the

strips were eluted overnight in 2 ml water, after which time 1 ml was removed and counted as above.

Preparation of Synthetic AcNeu7/[^{3}H]-AcNeu7

AcNeu7 was synthesized from synthetic N-acetyl-neuraminic acid (NeuAc) which was purchased from Sigma. A 3 mM solution of $NaIO_4$ was allowed to react with 2 mM NeuAc for 1 h at 37 °C in a total volume of 5 ml, at which time 0.5 ml of 10% glycerol was then added to react any $NaIO_4$ remaining. A sample was tested for any residual $NaIO_4$ by reacting 0.1 ml of the reaction mixture with 0.5 ml of 0.25 M sodium-m-arsenite while monitoring the above solution at 500 nm in a Gilford single-beam recording spectrophotometer. An increase in the absorption of the solution is observed if unreacted $NaIO_4$ is still present. Glycerol was added dropwise in such cases until the solution remained clear upon the addition of arsenite. After reduction with 5 mM $NaBH_4$ for 30 min at room temperature, the solution was spotted on 4-inch wide strips of Whatman 3 MM paper and chromatographed in solvent system B for 15 h. A 0.5 cm strip was then cut from the center of the chromatogram and after elution the sugar was located on this strip by the pyridoxamine assay.

[^{3}H]-AcNeu7 was produced by substituting $NaB[^3H]_4$ in the above reaction for $NaBH_4$. After reduction of the

mixture, it was spotted and chromatographed as above. A one-half cm strip was again cut from the center of the chromatogram and analyzed for both radioactivity and pyridoxamine reactive AcNeu7. The peak corresponding to [^{3}H]-AcNeu7 was eluted and rechromatographed twice to isolate labeled monosaccharide.

Preparation of the Total Particulate Fraction

The total particulate fraction was isolated from cells which were homogenized as was described for the isolation of plasma membranes (Atkinson, 1973; Pollack et al., 1981a). After the addition of the divalent ions to stabilize the nuclei, one-third of the cell homogenate was placed in a 1.5 ml Eppendorf tube and then centrifuged for 5 min in a Brinkman Micro Centrifuge, Model #5412. The particulate fraction was washed twice with cold PBS and assayed for protein and radioactivity. When labeled as described above, 95% of the cell associated radioactivity is sedimented by this procedure. In addition, only about 2% of the cell-associated radioactivity in HTC or HeLa cells is acid soluble 18 to 24 h after labeling (data not shown). The remaining two-thirds of the original cell homogenate was used to isolate plasma membranes, as was described (Pollack, et al., 1981a).

Determination of Cell-Associated Acid-Soluble Radioactivity

Approximately 10^7 cells labeled as above were assayed for TCA-soluble radioactivity after being washed three times and resuspended in 1 ml of ice cold PBS. The cells were homogenized in a tight fitting Dounce and sonicated for 30 s at 100 W with a Braunsonic 1510 sonicator. One ml of 20% TCA was added and the homogenate incubated for 60 min on ice followed by a 95% ethanol extraction and an ether extraction under the same conditions. The TCA-precipitable material was then washed twice, the pellet solubilized in 0.1 N NaOH, neutralized with 1 N HCl, and the radioactivity determined. The TCA-solubilized material and washes were pooled and their radioactivity determined.

Results

Neuraminidase digestion was performed on HTC or HeLa cells labeled as described above. About 77 or 85% of the surface-associated radioactivity was removed from HTC or HeLa cells, respectively, as judged by analysis of plasma membranes isolated from these cells before and after neuraminidase treatment (see below). 5'-Nucleotidase, a

stable plasma membrane marker (Brake, et al., 1978), is unaffected by exposure of cells to neuraminidase (Emmelot and Bos, 1965; and data not shown), and was used to monitor the yield of plasma membranes. The specific activities of surface markers were enhanced 8-12 fold in the isolated membranes from both cell types.

Analysis of Radioactive Material from Labeled Cells

Plasma membranes from labeled HTC and HeLa cells were analyzed by SDS-PAGE (Figure 1). Fluorography of the dried gels revealed numerous labeled glycoproteins present in both HeLa (lane 1) and HTC (lane 3) cells. No bands with significant label were seen in plasma membranes from cells that had been treated with neuraminidase, i.e., no detectable labeled surface glycoproteins were specifically insusceptible to the enzyme.

Analysis by paper chromatography of the labeled product released by acid hydrolysis (data not shown) or neuraminidase digestion of surface labeled HeLa cells revealed a single major peak of radioactivity (Figure 2). This peak was seen to migrate between sialic acid and glucose. Previous investigations have demonstrated the radioactivity is stably associated with a seven carbon derivative of sialic acids, termed AcNeu7 (Van Lenten and

Figure 1. Fluorographic analyses from sodium dodecyl sulfate- polyacrylamide gel electrophoresis of enriched plasma membrane fractions from HeLa-S3 and HTC cells after treatment with $NaBH_4/NaIO_4/NaB[^3H]_4$. Plasma membranes were from HeLa cells (1), HeLa cells treated with neuraminidase (2), HTC cells (3), and HTC cells treated with neuraminidase (4). Conditions are outlined under Material and Methods. BPB, bromophenol blue tracking dye.

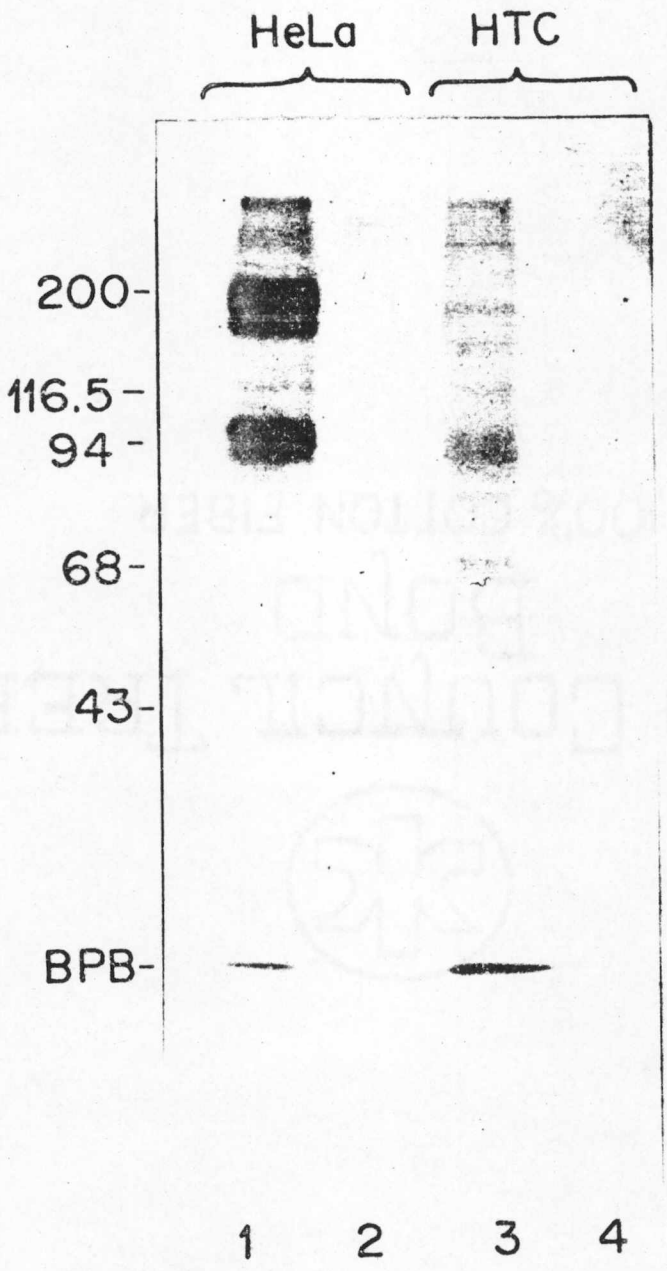


Figure 1

Ashwell, 1971; Gahmberg and Andersson, 1977; Dodeur and Bourillon, 1978). The chromatographic profiles of the radioactivity released from the cells by neuraminidase were identical regardless of the length of incubation of the labeled cells (Figure 2). Thus, the surface-labeled AcNeu7 is not modified by the cell. In addition, the fraction of radioactivity on the cell surface susceptible to release by neuraminidase remained at 77 and 85% for HTC and HeLa cells, respectively, independent of the length of incubation of the cells after labeling. A typical experiment with HeLa cells is summarized in Table I.

Continuous exposure of either HTC or HeLa cells for 48 h to [^{14}C]-glucosamine, a biosynthetic precursor of sialic acids, yielded no metabolically labeled, yet neuraminidase-sensitive residue comigrating with AcNeu7 (data not shown) yet there was substantial amounts of radioactive material comigrating with authentic sialic acid was released by the enzyme. If the sialic acids of these cells were converted to AcNeu7 as described above after metabolic labeling, the ^{14}C radioactivity released by neuraminidase comigrated on paper chromatography with AcNeu7 prepared as above or that prepared synthetically (data not shown).

Figure 2. Chromatographic pattern of cell-associated, neuraminidase-sensitive radioactivity at various times after $NaBH_4/NaIO_4/NaB[^3H]_4$ treatment. The neuraminidase-sensitive radioactivity, which was released from HeLa cells in the experiment outlined in Table I, was analyzed by paper chromatography in solvent system A. Absolute values for counts/min in the peak fractions were: 0 h, 650; 18 h, 690; 42 h, 350; 66h, 245.

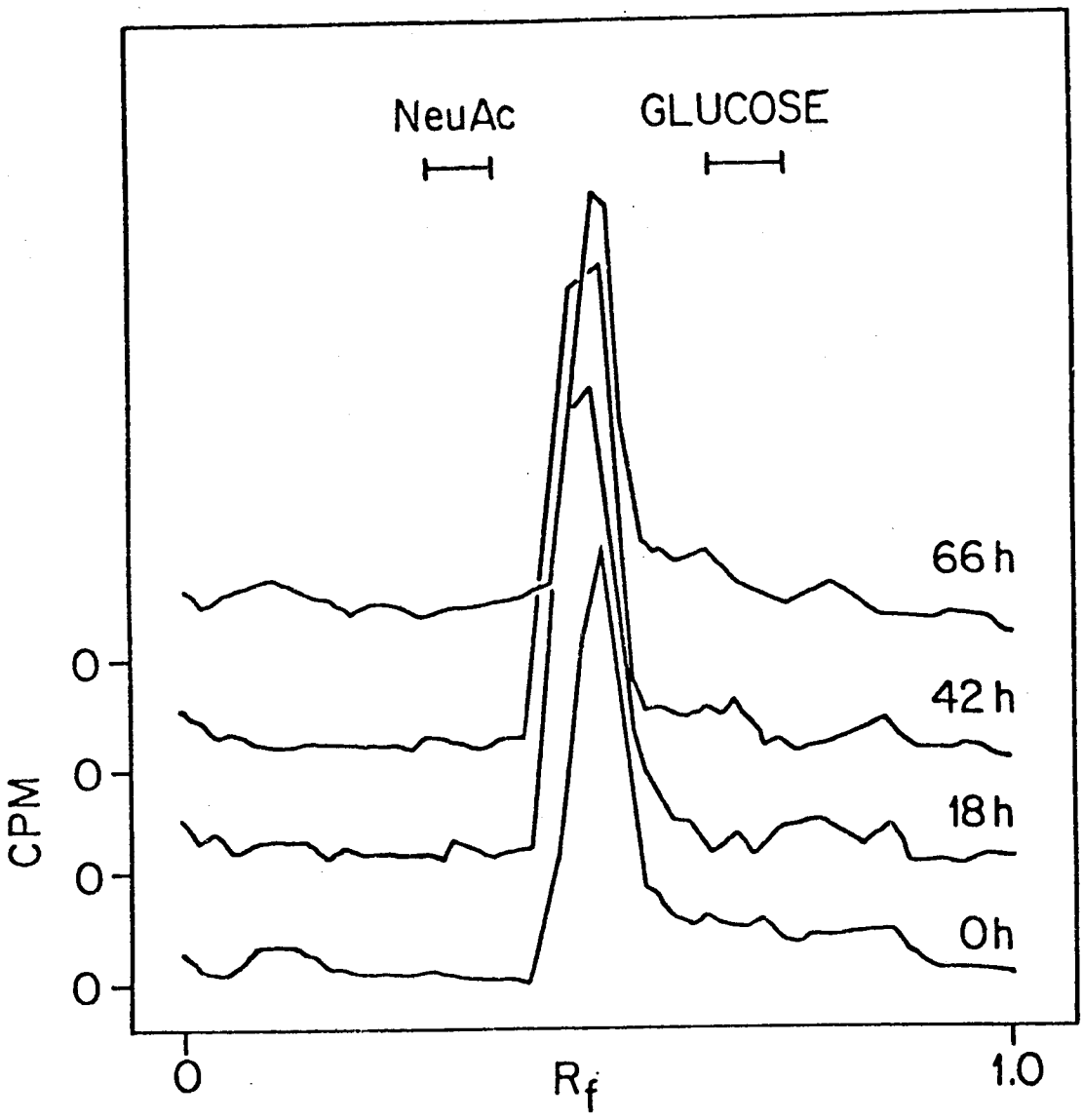


Figure 2

TABLE I

Neuraminidase sensitivity of plasma membrane-associated radioactivity

$NaBH_4/NaIO_4/NaB[^3H]_4$-treated HeLa cells (3×10^8) were washed exhaustively and resuspended in 750 ml of growth medium containing 0.1 mM AcNeu7. At the times indicated, plasma membranes were isolated before (-N'dase) and after (+N'dase) neuraminidase treatment of the cells. Plasma membranes were analyzed for protein, radioactivity, and 5'-nucleotidase activity.

Hours after labeling	Counts/min/mg protein			Counts/min/unit 5'-nucleotidase*		
	(-N'dase)	(+N'dase)	% released	(-N'dase)	(+N'dase)	% released
1	63,921	12,021	82	23,244	4,145	82
18	16,851	2,359	86	6,018	842	86
30	4,672	701	85	1,763	250	86
42	4,055	602	85	1,502	207	86
66	3,070	403	86	1,181	165	86

*1 unit = 1 mol of [^{14}C]adenosine released from [^{14}C]AMP per mg protein per h.

Loss of [^{3}H]-AcNeu7 From HTC and HeLa Cells

To determine the suitability of using the above labeling technique in various long-term assessments of recycling, the turnover of [^{3}H]-AcNeu7 containing macromolecules was measured (Figure 3). A biphasic loss of radioactivity was seen. If the curves are fit as the sum of 2 exponentials, without any further implications or assumptions with respect to mechanism, the fast phase of loss had a t/2 of 4-5 h, and the slow phase a t/2 of 100-200 h in both HTC and HeLa cells. Similar turnover rates had been observed in these cell types using other methods of labeling (Huang et al., 1973; Baumann and Doyle, 1978). AcNeu7 is not reutilized by either cell type (see below).

Temporal Sensitivity of Cell-Associated [^{3}H]-AcNeu7 to Hydrolysis by Neuraminidase

The time-dependent loss of cell-associated radioactivity from both HTC and HeLa cells was compared to the time dependent change in radioactivity sensitive to removal by extracellular neuraminidase (Figure 4). Although the loss of total cell-associated radioactivity was continuous (Figure 4A), the fraction of radioactivity sensitive to removal by extracellular neuraminidase

Figure 3. Loss of cell-associated radioactivity in HeLa and HTC Cells. $NaBH_4/NaIO_4/NaB[^3H]_4$-treated cells were resuspended at 2 X 10^5 in growth medium. At the times indicated, 1 ml of cells was pelleted in an Eppendorf Micro Centrifuge and washed with S-EMEM. The pellet was resuspended in 1.5 ml of cold 10% TCA, kept on ice for 20 min, and recentrifuged. The final pellet was dissolved in 1 ml of 0.1 N NaOH and neutralized and the radioactivity was determined. Each point is the average of duplicate determinations. Cells were diluted 1:1 with fresh growth medium 40 h after labeling and the radioactivity normalized to predilution values.

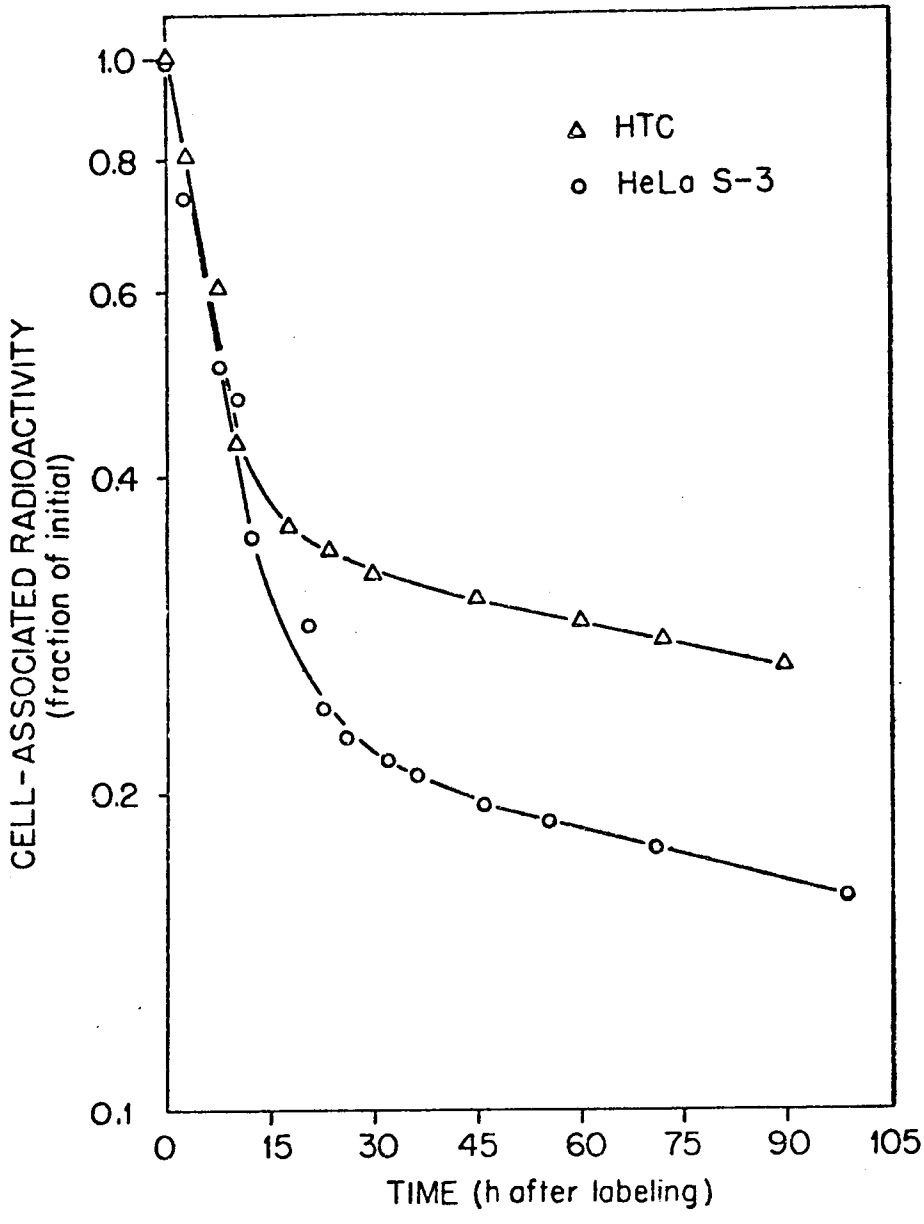

1.0
0.8
0.6
0.4
0.2
0.1
CELL-ASSOCIATED RADIOACTIVITY
(fraction of initial)
HTC
HeLa S-3
0
15
30
45
60
75
90
105
TIME (h after labeling)

Figure 3

Figure 4. Sensitivity of cell-associated radioactivity to removal by extracellular neuraminidase. A, see legend to Figure 3 for details. B, the neuraminidase-sensitive radioactivity expressed as the fraction of radioactivity present at each point in A. One h prior to analysis, 5 ml of cells are treated with neuraminidase as described in Material and Methods, except that the neuraminidase was added directly to the medium containing the growing cells and adjusted to pH 5.0 with 10 N HCl (diluted with S-EMEM) to avoid any perturbation of the cell surface observed with harvesting the cells via centrifugation. The specific radioactivity was then normalized to 1 ml of cell suspension prior to the addition of the neuraminidase and HCl.

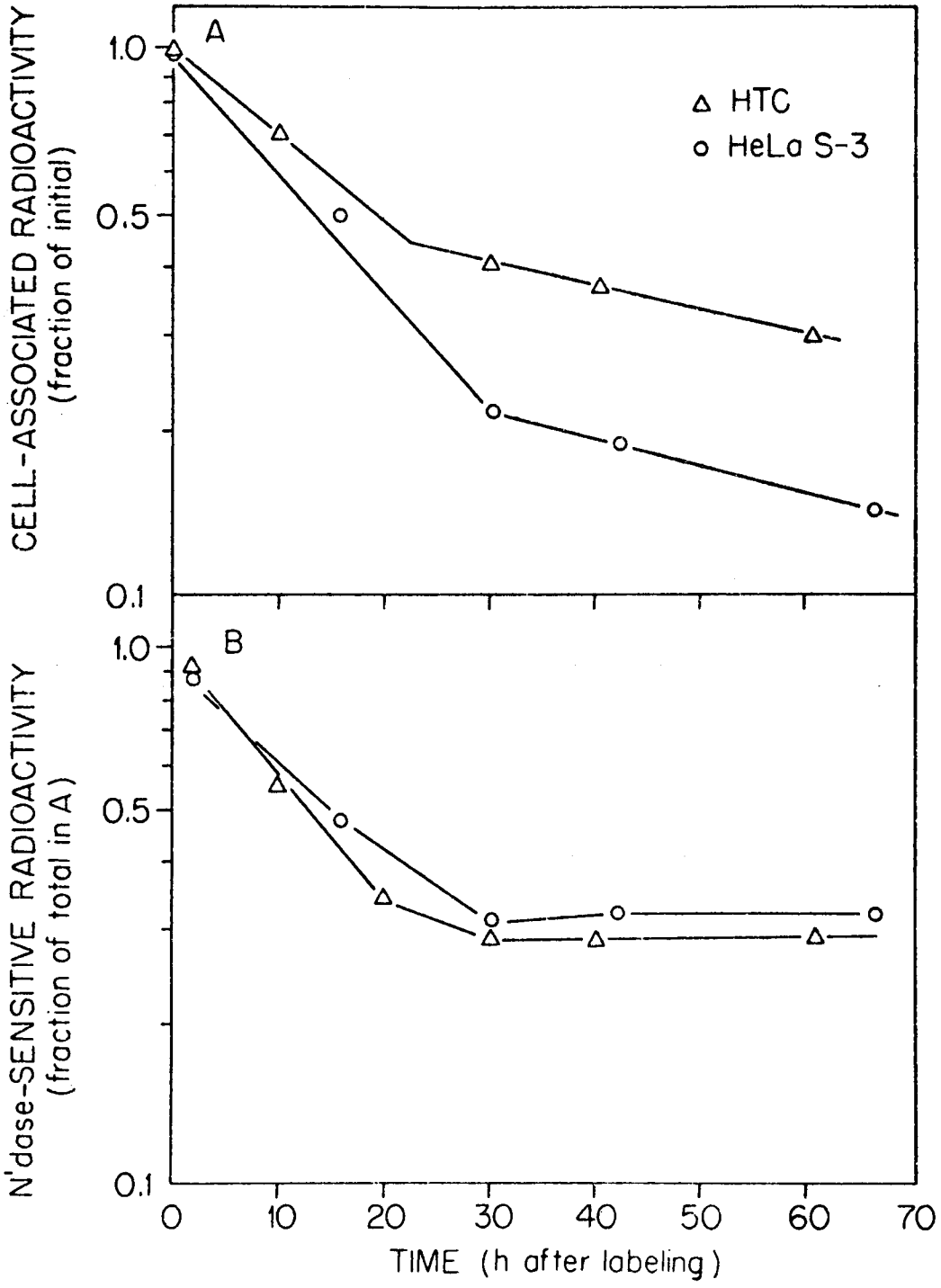

A
B
HTC
HeLa S-3
CELL-ASSOCIATED RADIOACTIVITY
(fraction of initial)
N'dase-SENSITIVE RADIOACTIVITY
(fraction of total in A)
1.0
0.5
0.1
0
10
20
30
40
50
60
70
TIME (h after labeling)

Figure 4

leveled off at about 30% of the total (Figure 4B). This relationship suggested a redistribution between the neuraminidase-insensitive pool of membrane constituents which was believed to be internal, and the cell surface (see Discussion). Thus, even though a continual loss of cell-associated radioactivity is observed, once a steady state is reached between the internal and external pools, no further change is observed in the fraction that is neuraminidase sensitive.

Bidirectional Flow of Labeled Plasma Membrane

The steady state value seen in whole cells of 30% neuraminidase sensitivity (Figure 4B) can also be achieved by creating an intracellular pool which has a specific radioactivity higher than that of the plasma membrane (Figure 5). The cell surface sialoglycoconjugates of HTC were labeled as was described above and resuspended for 8 hours in growth medium, thus allowing internalization of labeled plasma membrane to take place. The cells were then collected and subjected to a first neuraminidase (I) treatment to remove any sialic acids on the cell surface. The cells were then resuspended again in growth medium for varying amounts of time. Assessment of cell-associated radioactivity and neuraminidase-sensitive radioactivity on

Figure 5. Reappearance of internalized cell surface radioactivity on the external surface of HTC cells. HTC cells were labeled and subsequently analyzed for cell-associated and for neuraminidase-sensitive radioactivity as described in Figure 3, except that all cells remaining after an 8 h incubation were subjected to neuraminidase (I) treatment as described in Material and Methods (down arrow). Cells were then resuspended in growth medium (up arrow) and at the times indicated, aliquots of cells were assayed for their cell-associated radioactivity and neuraminidase-sensitive radioactivity. Main Figure: all values for neuraminidase-sensitive radioactivity were normalized to percent radioactivity of the whole cells at the time of sampling and represent the mean $\pm$ S. D. of triplicate samples. Inset: the total radioactivity associated with the cell samples after the neuraminidase treatment at 8-9 h after labeling.

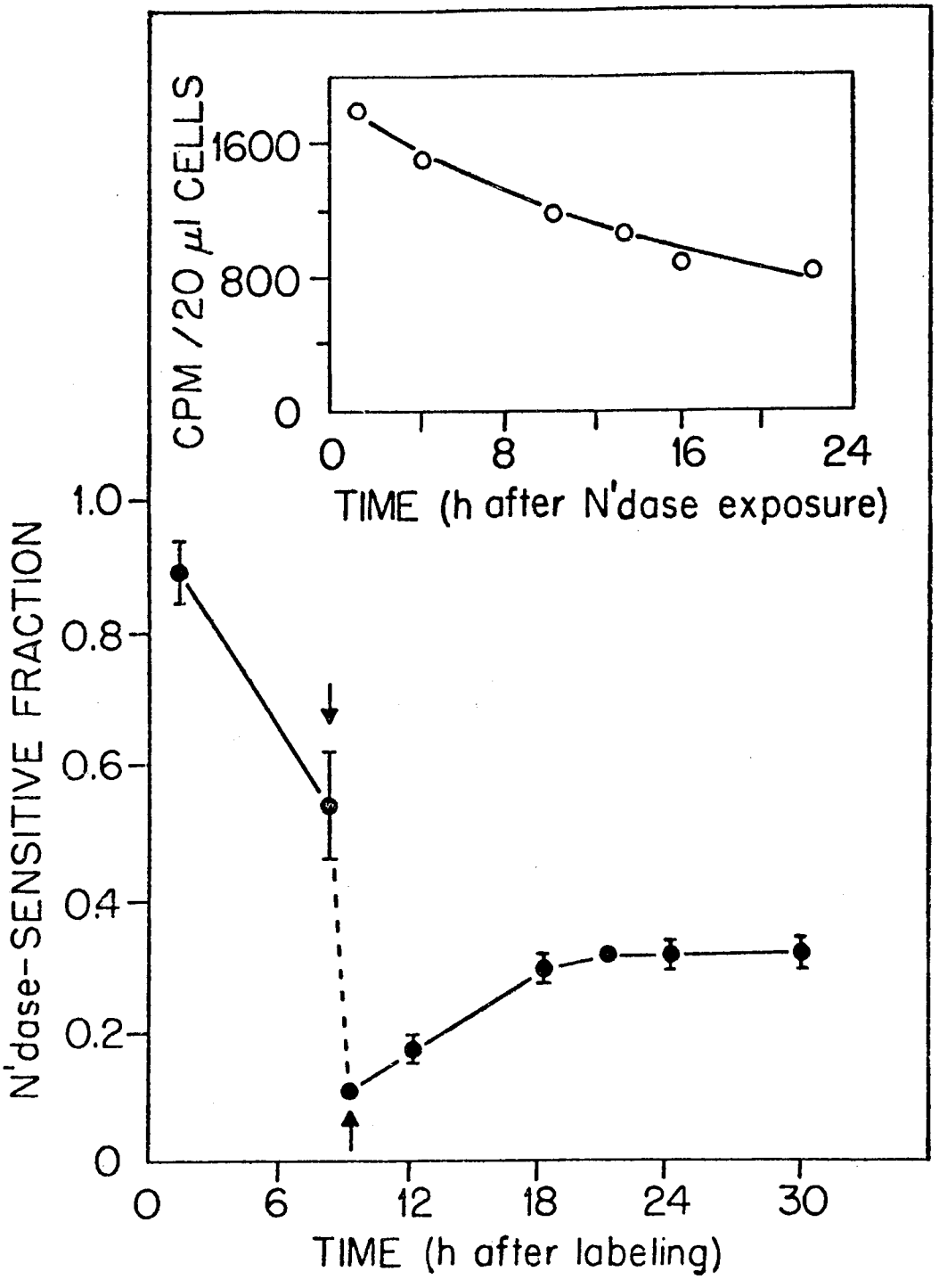

CPM /20 μl CELLS
1600
800
0
0
8
16
24
TIME (h after N'dase exposure)
N'dase-SENSITIVE FRACTION
1.0
0.8
0.6
0.4
0.2
0
0
6
12
18
24
30
TIME (h after labeling)

Figure 5

the cell surface was performed as described in the legend to Figure 4. Eight hours after labeling, neuraminidase (I) treatment left little (approximately 11% of the total cell-associated radioactivity) neuraminidase-sensitive radioactivity on the cell surface. Twelve hours after neuraminidase (I) treatment, approximately one-third of the total residual cell-associated radioactivity was again found to be sensitive to removal by a second neuraminidase (II) treatment, suggesting a return of label to the cell surface, and no significant change in this fraction was observed for the next ten hours, when the experiment was terminated. As the total cell-associated radioactivity decreased during the entire length of the experiment (see inset, Figure 5) there was no significant change in the fractional distribution of the neuraminidase-sensitive radioactivity after a steady state had been reached. Experiments with HeLa cells yielded similar results, as did HTC cells incubated 12 or 18 h prior to the first neuraminidase treatment. The 8 h incubation between the labeling and the first neuraminidase (I) treatment was used in most experiments because it minimized the loss of total cell associated radioactivity seen with prolonged incubation, yet provided a sufficiently large internal pool of radioactivity for observation of relabeling of the cell surface from the interior.

The redistribution observed for internalized [^{3}H]-AcNeu7-glycoconjugates as seen in Figure 5 was further investigated by comparing the specific radioactivity of the isolated plasma membranes ghosts to that of the total particulate fraction. Immediately after surface labeling (Figure 6, 0 h after labeling), there is a 5-fold higher specific radioactivity in the isolated plasma membranes then is seen in the total particulate fraction (nine-fold higher than was in the total cell homogenate; data not shown), which includes the plasma membranes. Eight hours after labeling, just prior to the neuraminidase (I) treatment, the specific radioactivity on the plasma mem- branes had decreased about three-fold while that of the particulates had decreased only slightly, the specific radioactivity on the plasma membrane being now only about twice that of the total particulate. Then, neuraminidase (I) treatment reduced the specific radioactivity on the above isolated plasma membranes by a further 80% (9 h after labeling in Figure 6), but this loss had a relatively smaller effect on the much larger pool from the total particulates. At 4 and 13 hours after neuraminidase (I) treatment (13 and 22 h after the intial labeling), the specific radioactivity of the particulate pool continued to fall slowly while that of the plasma membrane rose until it again exceeded the specific radioactivity associated with the isolated

Figure 6. Changes in the specific radioactivity of isolated plasma membranes and total cellular particulate fraction before and after neuraminidase treatment of HTC cells. HTC cells, labeled as described in Table I, were incubated in growth medium. Immediately and again at 8 h after labeling, aliquots were harvested and the specific radioactivity of isolated plasma membranes (shaded bars) was compared to that of the total cellular particulate fraction (open bars). The remainder of the cells were treated with neuraminidase as described in Material and Methods. Isolation of plasma membranes and the total particulate fraction was immediately performed on a small aliquot of the cells. After a 4 h or 13 h incubation in growth medium of neuraminidase-treated cells, plasma membranes and the total particulate fraction were again isolated. All values represent the mean of duplicate determinations.

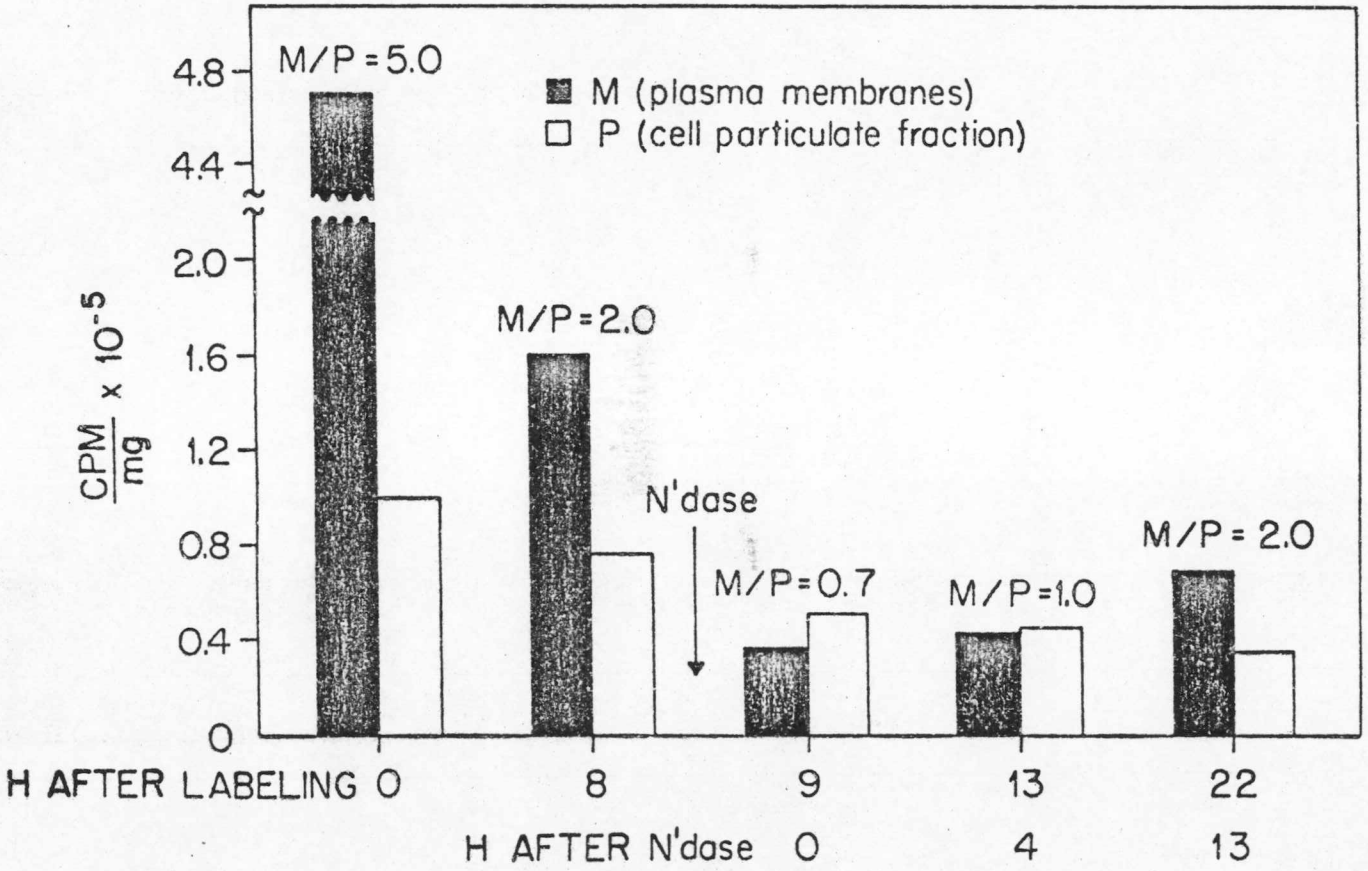

M/P = 5.0
M (plasma membranes)
P (cell particulate fraction)
4.8
4.4
2.0
1.6
1.2
0.8
0.4
0
CPM/mg x 10-5
M/P = 2.0
N'dase
M/P = 0.7
M/P = 1.0
M/P = 2.0
H AFTER LABELING 0
8
9
13
22
H AFTER N'dase 0
4
13

Figure 6

total particulate fraction. At 13 hours following neuraminidase (I) treatment the ratio of the specific activities returned to about the same value observed before neuraminidase (I) treatment. These data support the conclusions drawn from the previous experiment (Figure 5), that the label in the particulate fraction, apart from the surface membrane component itself, constitutes a pool from which label can return to the surface.

[^{3}H]-AcNeu7 Is Not Reutilized

Turnover of plasma membrane components, which was previously shown to be a slow process (Warren and Glick, 1968; Huang et al., 1973; Roberts and Yuan, 1974; Baumann and Doyle, 1978; Pollack et al., 1981b) could, nonetheless provide, by intracellular hydrolysis, a pool of AcNeu7 for incorporation into newly synthesized sialoglycoconjugates. To investigate the question of whether the above results may have been due to reutilization of labeled AcNeu7, HTC or HeLa cells were grown in medium contained 9.6 X 10^6 cpm per ml synthetic [^{3}H]-AcNeu7. After 48 h, a substantial quantity of [^{3}H]-AcNeu7 was found intracellularly, but none of this cell-associated radioactivity could be precipitated with TCA (Table II). If cells that had been allowed to take up [^{3}H]-AcNeu7 were

TABLE II

Lack of incorporation of synthetic [^{3}H]-AcNeu7 into trichloroacetic acid precipitable material of HTC and HeLa cells

HTC or HeLa cells were incubated in the presence of 2 mM [^{3}H]-AcNeu7 (≅4 uCi/mmol; 9.6 X 10^6 cpm/ml) for 48 h. Cells were then washed exhaustively with cold S-EMEM. After assay for cell-associated radioactivity, the cells were homogenized in a glass Dounce homogenizer and sonicated for 30 s prior to TCA precipitation. Values are average ± S. E. of triplicate determinations.

Cells	Cell-associated counts/min	Trichloroacetic acid-precipitable counts/min	% counts/min trichloro-acetic acid precipitable
HeLa	43,115 ± 408	49 ± 19	0.12
HTC	26,340 ± 766	78 ± 12	0.30

subsequently resuspended in fresh growth medium, 95% of the radioactivity was found in the extracellular medium within 12 h, and all of it was chromatographically indistinguishable from the monosaccharide AcNeu7.

As an additional control, experiments like those shown in Figures 4 and 5 were performed with 0.1 M AcNeu7 in the medium throughout, making available to the cells a source of unlabeled AcNeu7 which could dilute the pool of potentially reutilizable [^{3}H]-AcNeu7. In spite of the presence of this unlabeled AcNeu7, no differences were observed in the results.

Discussion

Internalization of the cell surface is a very rapid process in a variety of cell types (Steinman et al., 1976). The turnover of membranes in these same cell types, however, is one to two orders of magnitude slower than the observed rate of internalization (Steinman et al., 1976). As outlined in Chapter One, such disparity could be explained by the existence of a recycling pathway, whereby many internalized plasma membrane components are returned intact to the cell surface (Steinman et al., 1976; Tulkens et al., 1977; Louvard, 1980; Muller et al., 1980; Schneider et al., 1981b; Morre, 1981;

Widnell et al., 1982). This process spares the bulk of internalized material from degradation, thus sparing the cell extensive replacement biosynthesis of internalized macromolecules.

An analysis of cell surface recycling in both HeLa and HTC cells was performed using a minimally perturbing, non-permeating labeling technique. Sequential exposure of these cells to $NaBH_4$, $NaIO_4$, and $NaB[^3H]_4$ resulted in the specific modification of externally exposed sialic acid residues on surface glycoconjugates to a seven carbon derivative, termed AcNeu7 (Van Lenten and Ashwell, 1971; Gahmberg and Andersson, 1977; Dodeur and Bourillon, 1978). This sugar, although not a naturally occurring sugar residue in the cell, remained sensitive to cleavage by neuraminidase (Blumenfeld et al., 1972; and Figure 1 and Table I). Analysis of the enriched plasma membranes by SDS-PAGE and fluorography revealed the presence of numerous radioactive bands (Figure 1; at least 16-18 distinct radioactive bands are visible on the original autoradiographs). Neuraminidase treatment of labeled cells prior to electrophoresis and their analysis as above indicated that neuraminidase had removed the radioactivity from virtually all of the material which had entered the gel. Thus, neuraminidase, as used in this study, acted as

a nonspecific membrane probe, removing virtually all of the sialic acids and/or AcNeu7 from the cell surface (see below). In addition, incubation of the cells for varying times after surface labeling revealed no modification of [^{3}H]-AcNeu7 during the time course of these experiments (Figure 2) nor did it change its sensitivity to cleavage by neuraminidase with time, as was assayed in isolated membranes (Table I).

Coomassie blue stained gels of membranes from treated cells were indistinguishable from the control gels, and no new high molecular weight bands appeared after the above treatments (not shown). These observations suggested that no significant cross-linking of the proteins had taken place during the treatment.

The extent of both the initial labeling and its neuraminidase sensitivity depended on the cell type. The initial experiments were performed with HeLa cells, where the neuraminidase sensitivity of membrane-associated radioactivity was found to be 82 to 86%. In HTC cells, however, the neuraminidase sensitivity was somewhat less, varying between 75 and 79% of the surface radioactivity. In all other respects, the fate of the label in the two cell types was qualitatively very similar, varying only slightly in some of the quantitative aspects as noted above.

Since the label on the plasma membrane was not 100% susceptible to enzymatic removal, an attempt was made to identify the insensitive component. The "nonspecific" labeling was estimated by treating the cells sequentially with $NaBH_4$ and $NaB[^3H]_4$, which resulted in a tritium incorporation equal to about 5% of that observed when the normal labeling procedure was used. Given that this 5% is background, and given further that the neuraminidase treatment was a limit digest removing 75-85% of the total label, there remained 10-20% of the surface radioactivity in periodate-sensitive, yet neuraminidase-insensitive residues, presumably sialic acids. This may have been due to the presence of neuraminidase-insensitive sialic acids (N-methoxy- or diacetyl-neuraminic acid; see also Gottshalk, 1960), or may have been simply the presence of some contaminating intracellular (i.e. neuraminidase-insensitive) material which was not separated from plasma membrane ghosts during the fractionation procedure.

The decrease in the fraction of cell-associated radioactivity that was sensitive to neuraminidase (Figure 4B) may have in principle reflected a change in the configuration of surface sialoglycoconjugates so that in some way the AcNeu7 became shielded from the action of neuraminidase. This mechanism was ruled out by the data in Table I which showed that the sensitive fraction found

in membranes isolated after enzyme treatment of the whole cells remained constant throughout the experiment. The data in Figure 6 also supported the absence of cryptic sites within the plasma membrane, with the data for the radioactivity associated with isolated plasma membranes being virtually identical to what was obtained for the neuraminidase-sensitive radioactivity associated with the cell surface as shown in Figure 5. In addition, when the cells were incubated in the presence of 0.2% Triton X-100, a nonionic detergent, and neuraminidase, there was about a two-fold increase in the release of sialic acid over cells which were incubated without detergent (data not shown). Permeabilization of the cells with Triton thus allowed the digestion of intracellular sialoglycoconjugates to take place, suggesting that the conversion of sialic acids to AcNeu7 does not seem to significantly alter the sialoglycoconjugate partitioning between the plasma membrane and the intracellular pool.

The loss of neuraminidase-sensitive radioactivity from the surface of these cells could be attributed to three phenomena acting simultaneously: (1) There may have been direct loss from the cell surface into the medium by shedding (Black, 1980) or degradation directly at the cell surface via serum and/or cell surface proteases. The early rapid phase of loss (Figures 3 and 4A) may be in

part due to shedding. By gel filtration analysis much of the radioactivity which appeared in the medium during this early phase had a molecular weight on the order of 0.5-2 X 10^6, suggesting that it was in a particulate rather than macromolecular form; (2) Highly labeled external membrane exchanged with an initially unlabeled intracellular pool. The argument for this recycling processs is developed more fully below; and (3) The degradation of a portion of the internalized surface membrane, or turnover, which was observed to occur at a slow rate (Figures 3 and 4A; Huang et al., 1973; Doyle et al., 1978; Tweto and Doyle, 1979; Doyle and Baumann, 1980; Pollack et al., 1981b). This final slow phase of loss of cell-associated radioactivity presumably reflected this turnover, and the material that had appeared in the medium at that time was largely of the size of monomeric AcNeu7, as determined by gel filtration on Biogel P-2 (data not shown). Interestingly, near the end of the fast phase of release when the majority of the label was internal, only about 2% of the cell-associated label was acid soluble (data not shown). This suggested that when the sialoglycoconjugate was degraded, the AcNeu7 was lost rapidly from the cells.

The argument for recycling of internalized surface membrane presupposes the existence of an intracellular pool and the continuous exchange of materials between the

cell surface and this internal pool. The experiments shown in Figure 4 were designed to detect the labeling of the internal pool from the cell surface. Thirty hours after labeling of the cell surface, the fraction of the cell-associated radioactivity susceptible to removal by neuraminidase had fallen to about 30%, where it remained until termination of the experiment (66 h after labeling). The result suggested that this surface activity was exchanging with an internal membrane pool approximately equal in size to two cell surface equivalents. This conclusion was qualitatively substantiated by the data at 8 and 22 h in Figure 6. The isolated plasma membrane fraction was about 10-fold enriched in specific activities of 5'-nucleotidase and surface-bound ouabain compared to cell homogenate, i.e. contained about 10% of the cell protein. The total particulate fraction contained about 60% of the cell protein (data not shown) including both the plasma membranes and the internal pool. When the internal pool was twice the size of the external pool and at specific activity equilibrium with it, then the specific radioactivity of the total particulate fraction should have been about half that of the membrane fraction. This was what was observed.

Evidence for exchange between the internal and external pools was obtained by using two neuraminidase

treatments spaced varying lengths of time apart (Figures 5 and 6). After incubating the cells which allowed for the internalization of a substantial portion of the cell surface label, the remaining neuraminidase-sensitive radioactivity was enzymatically removed from the cell surface. Since neuraminidase removed approximately 75-79% of the cell surface radioactivity from the HTC cells, and Figure 4B gives a steady state value of around two cell surface equivalents located inside the cell, then the radioactivity in the total particulate fraction should have decreased by about one-fourth if the neuraminidase was hydrolyzing only surface sialoglycoconjugates. The radioactivity associated with the total particulate fraction was decreased by 27% after neuraminidase (I) treatment, consistent with the above argument that the neuraminidase was not being internalized by these cells and hydrolyzing intracellular sialoglycoconjugates. In addition, the adjustment of the extracellular pH to approximately 5.0 during the neuraminidase treatments inhibited the rate of fluid-phase endocytosis by greater than 90%, as was judged by the uptake of [^{14}C]-sucrose, further suggesting that little, if any, neuraminidase was entering the cells. This manipulation left relatively little radioactivity on the cell surface but did leave a comparatively large pool of radioactivity that was located

intracellularly. With further incubation, radioactivity returned to the cell surface from the internal pool, again achieving the 30% neuraminidase-sensitive plateau in whole cells.

Doyle et al. (1978) demonstrated in HTC cells an internal pool of membrane-associated fucoproteins that were in communication with the surface membrane. The relative sizes of the internal pool and the surface were about the same as were described here. They found that following a pulse of [^{3}H]-fucose, the internal pool was initially labeled to a higher activity than was the cell surface. When chased with unlabeled fucose, the activity in the internal pool started to fall almost immediately while that in the plasma membranes continued to increase for several hours, thus showing a precursor-product relationship. Since the activity in the internal pool leveled off at about half the initial value it had at the start of the chase, the authors concluded that only half of the internal pool was thus contributing to the surface membranes. The results presented above suggest another interpretation of their observation: while the internal pool was contributing labeled glycoprotein to the cell surface, the cell surface was recycling label back to the internal pool. In this interpretation the leveling off represents the establishment of a steady state between the

surface and the internal pool. During normal turnover, glycoprotein-containing components of the membrane(s) were fed into the degradative pathway, but since this was slow (half-time of 100 h or more) compared to mixing by recycling (half-time of 8 h or less, Figures 5 and 6), the loss of radioactivity from both pools should have occurred with about the same rate constant. This is what Doyle et al. (1978) found.

Using immunolabeling methods, Louvard (1980), working with MDCK cells, and Widnell et al. (1982), working with rat fibroblasts, have shown that specific surface enzymes (aminopeptidase and 5'-nucleotidase, respectively) were cleared from the cell surface in a short period of time, but that the immunoglobulins with which the respective enzymes were tagged reappeared on the cell surface some time later. These results are a clear demonstration of the recycling of the antibody-labeled membrane markers. Antibodies, however, are clearly resistant to lysosomal degradation (Schneider, et al, 1979a) and there remains some question as to whether or not the recycling observed in these cases might have been due to protection of the surface by degradation-resistant immunoglobulins.

Since the membranes in these cells were in fact turning over and since the cells were being grown with concomitant net synthesis of new membrane, no conclusion

about recycling could have been drawn unless reutilization of AcNeu7 was ruled out. Two tests for reutilization were performed. First, nonradioactive AcNeu7 was prepared chemically from sialic acid, and experiments like those shown in Figures 4 and 5 were performed with the cells grown in the presence of 0.1 M AcNeu7. No change in the kinetics of exchange of radiolabeled surface material was observed (data not shown), as would have been expected if a radioactive precursor being used in the biosynthesis of sialoglycoproteins was being diluted by some unlabeled precursor. Second, otherwise unlabeled cells were grown for two days in the presence of [^{3}H]-AcNeu7. This allowed ample opportunity for the uptake of label by endocytosis, i.e., into the recycling pool, as well as by any other mechanism that might have been operating. At the end of this labeling period, the radioactivity per unit cell volume was about two-thirds that of the medium. Virtually none of this radioactivity was acid precipitable, and, on resuspension of the cells in fresh medium, 95% of the radioactivity was released from the cells in 12 h. It was therefore concluded that AcNeu7 was not a substrate for the normal biosynthetic pathway and therefore was not reutilized in the experiments described here.

The argument for recycling may be summarized as follows. Immediately after labeling, about 75 to 85% of

the surface radioactivity was found to be neuraminidase sensitive. Although this fraction did not change with time in isolated plasma membranes, in whole cells the fraction fell to a plateau level of about 30%, suggesting that the insensitive fraction had become internalized. If the membrane was then stripped of all labeled AcNeu7, it subsequently became relabeled to the same plateau level from the internal pool. Throughout, the label remained with AcNeu7. If the cells were then allowed to take up monomeric labeled AcNeu7 from the medium, the label was not being incorporated into TCA-precipitable material, suggesting that the cells could not metabolize the AcNeu7 and that reutilization could not have been responsible for the reappearance of labeled AcNeu7 on the cell surface from within the cell. The lack of any change in the neuraminidase sensitivity of cell surface radioactivity has several important implications in light of the evidence for recycling presented here: (1) Since the radioactivity which appeared on the cell surface was again neuraminidase sensitive, the AcNeu7 had returned as the terminal carbohydrate in the sialoglycoconjugate; and (2) Since cleavage of the bond between any residues of the glycoconjugate would have resulted in the loss of the terminal and nonreutilizable label, it follows, therefore, that the sialoglycoconjugates have recycled intact.

CHAPTER III

THE SEQUENTIAL TRANSFER OF INTERNALIZED, CELL SURFACE DERIVED MATERIAL THROUGH THE LYSOSOMES AND GOLGI COMPLEX IN HeLa CELLS

Introduction

Chapter Two outlined the observations that the plasma membrane of HeLa cells is internalized at a rate that far exceeds the rate of turnover of cell surface components, and that much of this material is returned intact to the cell surface rather than degraded.

The time course of recycling of this material makes it clear that the data represent some kind of long-term recycling, in contrast to that observed for transferrin receptors (Bleil and Bretscher, 1982), which recycles in about 21 minutes or the asialoglycoprotein receptor which recycle in 5-15 minutes (Harford et al., 1984). In this chapter, several cellular fractionation procedures were developed to analyze the internalization of material from the cell surface through various organelles, to determine which intracellular organelle(s) this surface-derived material is associated with, and the order in which this association occurs. It was hoped that the use of a cell-surface label instead of an adsorbed marker might aid in

the analysis of the movement of cell surface-derived material through various organelles. This goal was achieved as outlined below, and a route for the movement of material from the cell surface through various intracellular organelles was established.

Materials and Methods

Cell culture conditions, the isolation of plasma membrane ghosts, and the assay of both protein and β-hexosaminidase were as described in Materials and Methods in Chapter II.

Chemicals

High specific activity $NaB[^3H]_4$ (15,000-20,000 mCi/mmol) was from Research Products International. Percoll was purchased from Pharmacia. Uridine diphosphate (U-[^{14}C])-galactose (UDP-galactose) was from New England Nuclear. Fluorescinated dextran was purchased from Sigma. All other chemicals were of the highest grade commercially available (see also Materials and Methods, Chapter Two).

Assay of Galactosyl Transferase Activity

Galactosyl transferase was assayed by a modification of the technique of Bretz and Staubli (1980). The various fractions were isolated as described below, and these

subcellular fractions to be assayed were incubated in the presence of sodium cacodylate buffer (pH=6.6), $MnCl_2$, and 2-mercaptoethanol, each at a concentration of 40 mM. Uridine diphosphate (U-[^{14}C])-galactose (UDP-galactose) was diluted to a specific activity of 0.2 mCi/mmol. Unlabeled UDP-galactose was present at 2.1 mM, and 18 mg/ml ovomucoid was added as an acceptor. Triton X-100 (0.25%) was present to yield maximum enzyme activation. Fractions isolated during centrifugation or from Percoll gradients were added to an equal volume of the solution above (total volume 0.1 ml) and assayed at 37°C for 15 to 60 minutes. The reaction was then terminated via direct addition of 1% phosphotungstic acid in 0.5 N HCl (PTA) to the reaction tube, and the mixture centrifuged, with the pellet washed three times with 10% TCA, once with 95% ethanol, and once with anhydrous ether. The pellet was dissolved in 0.2 N NaOH, neutralized with 1 N HCl, and counted in 10 ml of ACS scintillation fluid.

Labeling of Cells

HeLa cells were labeled as described in Chapter Two, with minor modifications. Briefly, HeLa cells (0.5-2 X 10^8) were washed 2X in S-EMEM, resuspended in PBS at a concentration of 5-10 X 10^6 cells/ml and treated for 5 min

at room temperature with 2 mM $NaBH_4$. The cells were washed, resuspended again, and exposed to 2 mM $NaIO_4$ for 10 minutes on ice. Then, 0.2 ml of 10% glycerol in glass-distilled H_2O was added to react any remaining $NaIO_4$. After five minutes, the cells were diluted with 20 vol of S-EMEM, centrifuged, and resuspended in a volume of 3 ml. The cells were reduced with 25 mCi of $NaB[^3H]_4$, swirled, placed quickly in a small flask on ice for 15-20 min, and finally reacted with 1 mM $NaBH_4$ to reduce any remaining aldehydes. The cells were then washed until the background radioactivity became insignificant. The efficiency of this washing procedure was improved by layering cells on top of 150 ml of 4% sucrose, 0.5X S-EMEM, and centrifuging the cells at 1500 RPM for 8 min in an IEC rotor number 284.

Neuraminidase Treatment of Cells

Labeled HeLa cells were resuspended in S-EMEM plus 4 mM glutamine at pH 5.0 containing 2 units/ml neuraminidase from Clostridium perfringens. The cells were incubated at 37°C for 60 min in a 500 ml glass bottle swirling at 110 cycles/min. Cells were washed twice with ice-cold S-EMEM prior to further use.

Percoll Gradient Analysis of Cells

Approximately 2 X 10^7 HeLa cells were homogenized in 0.25 M sucrose-10 mM Tris buffer using a loose-fitting Dounce to approximately 80% disruption. A post-nuclear supernatant was isolated via centrifugation of the Dounced cells for 15 min at 1500 RPM in an IEC centrifuge, rotor number 235, resuspending the pellet in 0.25 M sucrose and recentrifuging it twice at 1200 RPM, 10 min. The above supernatants were pooled and added to Percoll to give a final concentration of 22.5% Percoll-0.25 M sucrose, and centrifuged at 17,000 RPM for 30 min in a 50Ti rotor. The gradient was fractionated from the bottom into 25-0.4 ml fractions.

Dextran Alteration of Organelle Density

Cells to be used in the isolation of lysosomes or used in experiments designed to alter the density of Golgi fractions were incubated post-labeling with Dextran 500 (Sigma; Mol. Wt. = 5 X 10^5). Stock solutions (100X) were made up in S-EMEM, sonicated prior to use, and then filter sterilized. Alteration of the density of Golgi fractions was achieved by preincubating cells for 8-12 hours in the presence of 0.4 mg/ml dextran 500 prior to labeling and analysis. The procedure for isolating dextran-loaded lysosomes is oultined below.

Isolation of Golgi Fractions

Enriched Golgi fractions were isolated via use of a modification of the technique of Ehrenreich et al. (Bretz et al., 1980). Briefly, 1 X 10^8 HeLa cells were washed, homogenized as above, mixed with an equal volume of 0.6 M sucrose, and then centrifuged at 9100 RPM for 10 min in a Sorvall SS-34 rotor. The supernatant is then centrifuged at 37,500 RPM for 90 min in a 60 Ti rotor, the pellet collected and resuspended in 7.5 ml of 1.22 M sucrose. This material was layered under 7.5 ml each of 0.25 M, 0.6 M, 0.86 M, and 1.15 M sucrose, centrifuged at 23,600 RPM for 210 min at speed (brake off) in an SW28 rotor, and the 0.25/0.6, 0.6/0.86, and 0.86/1.15 M sucrose interfaces collected. In contrast to the situation reported for rat liver, the upper interface did not contain significant amounts of material. Also, the purification of the material pooled from the other two interfaces was only 4-6 fold over homogenate values. Thus, a second gradient was used to further purify this material, as described by Khan et al. (1982). Briefly, the two interfaces were pooled, adjusted to approximately 0.25 M sucrose, and added to Percoll to give a final concentration of 17% Percoll-0.25 M sucrose. This mixture was centrifuged in a 60Ti rotor at 16,700 RPM for 30 min at speed, and fractionated into

12-2 ml fractions. The fractions were assayed as shown in Figure 9, below.

Isolation of Lysosomes

Two procedures were used to isolate an enriched lysosomal fraction. The first was adapted from the method of Thines-Sempoux as described (Beaufay, 1972). Cells are incubated for three hours prior to labeling with 0.4 mg/ml dextran 500, homogenized as above and then a post-nuclear supernatant isolated. An intermediate-speed pellet (6000 RPM, 8 min, SS-34 rotor), was then resuspended in 15 ml of 33.5% sucrose (w/w) and layered over 5.5 ml each of 46.8%, 57.4%, 67.3%, and 62%(in D_2O) sucrose. This gradient was centrifuged for 120 min at speed, 26,000 RPM in an SW28 rotor, and the pellet, containing an enriched lysosomal fraction recovered. In the second method (Trouet, 1964), the post-nuclear supernatant from cells which had been incubated in the presence of 0.02 mg/ml Triton WR-1339 for 24 h was centrifuged at 24,000 RPM for 6 min at speed in a 60Ti rotor. The pellet contained an upper layer of light colored material, which was carefully aspirated off and a darker, lower layer was resuspended in 0.25 M sucrose and recentrifuged. The darker, bottom layer was again recovered, resuspended in 45% sucrose (w/w; 15 ml). This was layered under 4 ml of 14.3% sucrose (w/w), 6 ml of

31.3% (w/w), and 8 ml of 34.5% sucrose (w/w). Finally, 4 ml of 60.7% sucrose (w/w) was layered under the above solutions. This gradient was centrifuged for 120 min at 25,000 RPM in an SW28 rotor. The interfaces were then collected and analyzed for lysosomal enzyme activity (β-hexosaminidase). It was found that the 31.3%/34.5% sucrose interface yielded the purest lysosomes, having an 18 to 23 fold purification of β-hexosaminidase over homogenate.

Measurement of pH in Endocytic Vesicles and Lysosomes

Fluorescein conjugated to dextran (Sigma; Mol. wt. = 70,000) was used to estimate the pH of endocytic vesicles and lysosomes. Cells were plated onto small pieces of microscope slides and were allowed to grow almost to confluency. The cells were then washed in cold PBS and incubated in PBS containing 1 mg/ml fluorescein-dextran (FD) for 10 min at 18°C. Cells were then washed three times in EMEM at 18°C, and incubated in this medium for 10 min during observation in a Hitachi fluorometer. Cells were excited at 450 and 495 nm with emission analysis at 519 nm as was described (Ohkuma and Poole, 1978). Any cellular autofluorescence was estimated by monitoring the fluorescence as above of cells that were not exposed to FD and was subtracted. The medium was then aspirated off and

37°C medium was added and fluorescence monitored every 5 min. The temperature was maintained at 37°C via the incubation of the cuvettes in a constant temperature block in the dark between measurements. A standard curve was constructed with FD buffered at various pH (see inset, Figure 12).

Results

Percoll Gradient Analysis of Cell Surface Sialoglycoconjugates

The treatment of HeLa cells with $NaBH_4$, $NaIO_4$, and $NaB[^3H]_4$ results in cell surface sialic acid residues being modified to a seven carbon, radiolabeled analogue, AcNeu7 (Van Lenten and Ashwell, 1972). As discussed in Chapter II, this labeling technique allows analysis of sialoglycoconjugate recycling with a minimum of cellular perturbation. As was detailed in Chapter II, during steady-state conditions, the cell surface in HeLa cells is exchanging with an intracellular pool (halftime of 4-6 h) twice as large as the cell surface and from which the internalized surface material can recycle back to the cell surface. An analysis of this intracellular pool follows below.

Density gradient analysis was performed on HeLa cells labeled as above and incubated at 37°C for varying times

after labeling. A post-nuclear supernatant from HeLa cells Dounced in isotonic sucrose was added to 22.5% Percoll-0.25 M sucrose and centrifuged as described. The characteristics of the resultant gradients are shown in Figure 7 (a-d). Both the enzyme marker for the Golgi complex, galactosyl transferase, and the enzyme marker for the lysosomal compartment, β-hexosaminidase, sediment as distinct peaks at densities of approximately 1.045-1.055 g/cc and 1.09-1.11 g/cc, respectively. The position of the plasma membrane material is indicated by the peak of radioactivity at density 1.03 g/cc from cells harvested immediately after labeling. When cells are incubated after labeling and analyzed as above (Figure 7, e-j), the radioactive profile is altered with time.

During the first thirty minutes after labeling, some radioactive material appears in a region of the gradient which is slightly more dense than the plasma membrane. This density corresponds to that previously reported for endocytic vesicles and endosomes in Percoll gradients (Wolkoff et al., 1984). Upon further incubation, material is seen to cosediment with the lysosomal enzyme marker, β-hexosaminidase. One hour after labeling, there was approximately a 2-3 fold increase in radioactivity in fractions 2-8 over what was observed immediately after labeling. A second, major peak of radioactivity appearing

Figure 7. Percoll gradient analysis of cell surface labeled HeLa cells. HeLa cells were surface labeled using $NaBH_4/NaIO_4/NaB[^3H]_4$ and incubated for the times indicated. A post-nuclear supernatant was then isolated and the cells centrifuged in Percoll as described in Experimental Procedures. The fractions were then analyzed for density (a; using density marker beads from Pharmacia), galactosyl transferase activity (b), B-hexosaminidase activity (c), and radioactivity (d-g).

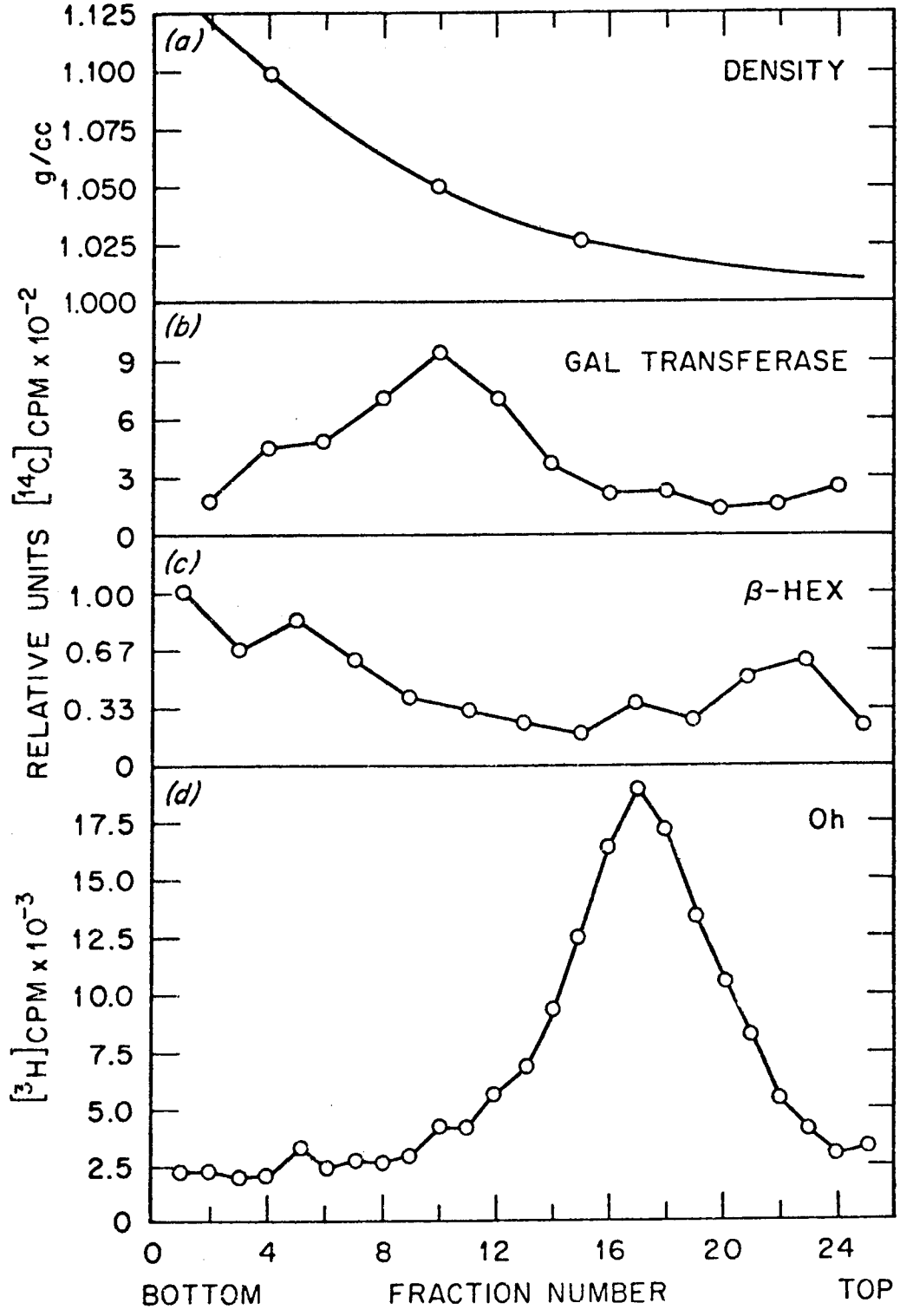

(a)
DENSITY
g/cc
1.125
1.100
1.075
1.050
1.025
1.000
(b)
GAL TRANSFERASE
9
6
3
0
(c)
β-HEX
1.00
0.67
0.33
0
(d)
Oh
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0
RELATIVE UNITS [14C] CPM x 10-2
[3H] CPM x 10-3
0
4
8
12
16
20
24
BOTTOM
FRACTION NUMBER
TOP

Figure 7

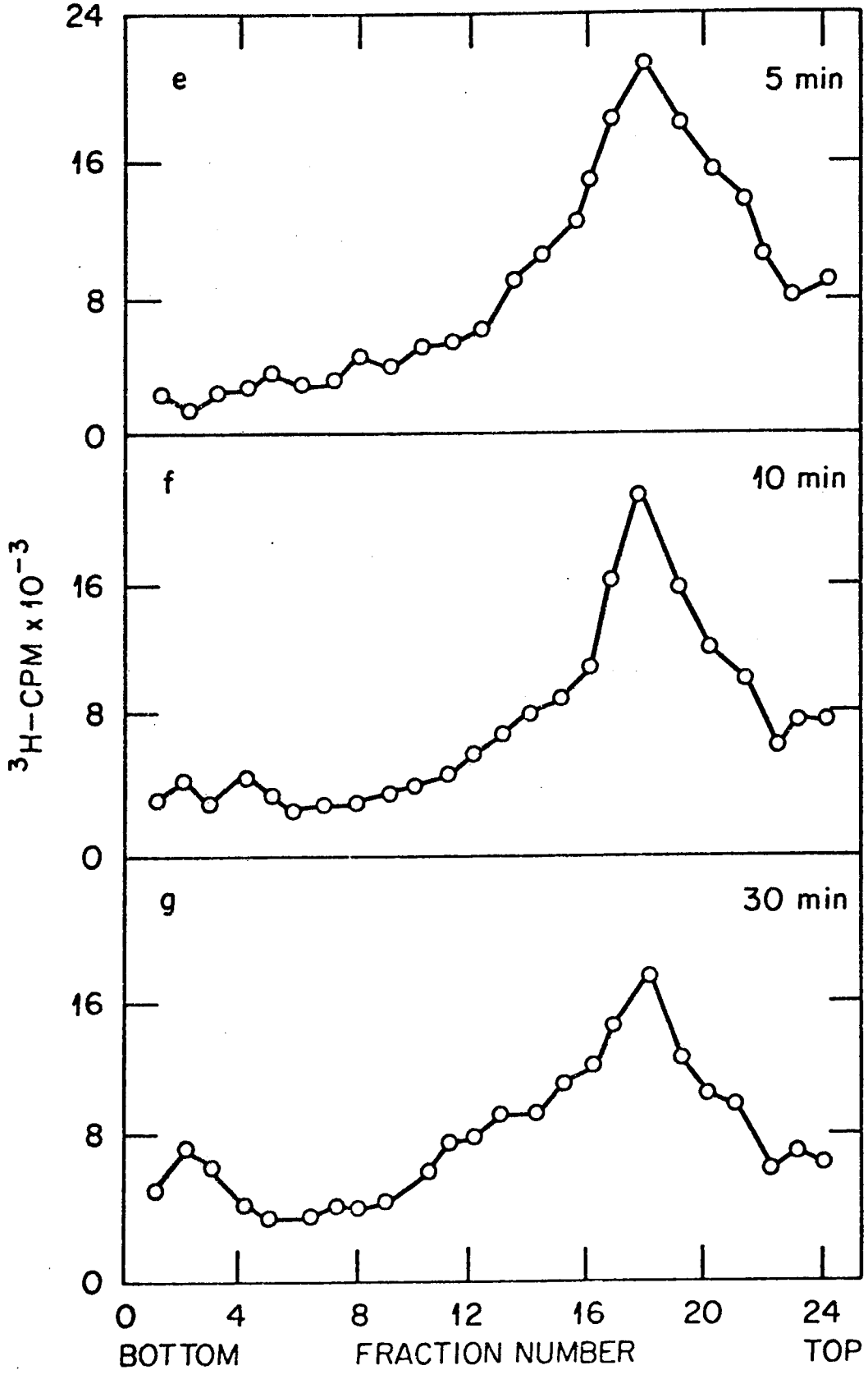


Figure 7, continued

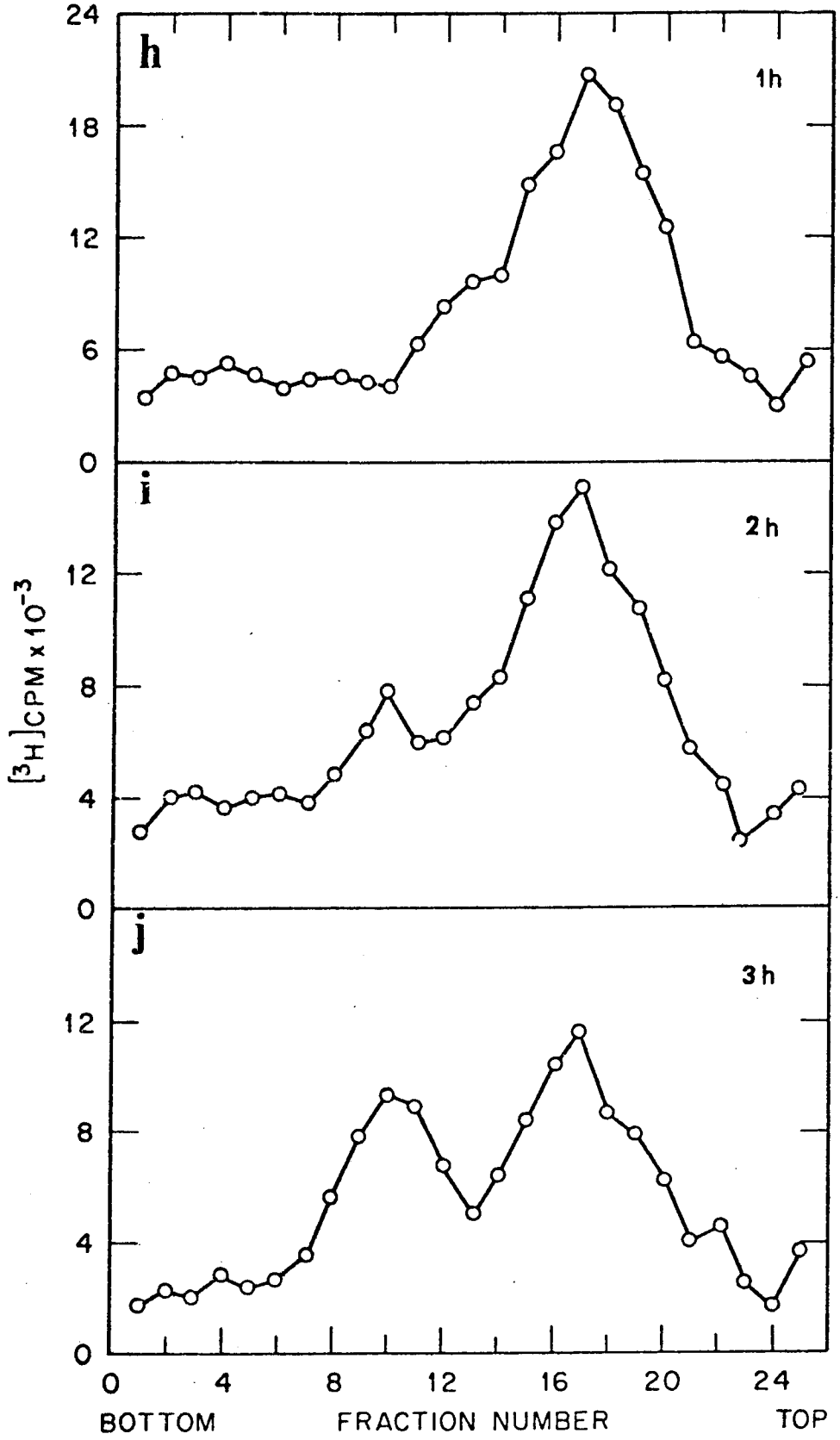


Figure 7, continued

between 1 and 3 hours after labeling (Figure 7, i,j) is codistributed with the Golgi complex marker, galactosyl transferase. No changes in the relative distribution of material in the various peaks is observed after 8 hours (data not shown).

The per cent of the total radioactivity associated with the lysosomes was estimated by treating cells with carefully controlled concentrations of digitonin. Cook et al. (1982) (see also Thines-Sempoux et al., 1969 and Tulkens et al., 1974) have previously shown that the treatment of a HeLa cell lysate with 0.03% digitonin solubilizes most of the particulate β-hexosaminidase activity, a lysosomal marker (Barrett, 1972), but only comparatively little 5'-nucleotidase activity, a plasma membrane marker. When similar experiments are performed with $NaBH_4/NaIO_4/Na[B^3H]_4$-treated cells (Table III), approximately 70% of the β-hexosaminidase activity is solubilized, compared to less than 20% of solubilized 5'-nucleotidase and ^3H immediately after labeling. When labeled cells were incubated for varying amounts of time after labeling and assayed for digitonin solubilization of markers as above, there is only a slight increase in the solubilization of the surface-derived radioactivity was observed. Since the above Percoll gradients demonstrated

TABLE III

Sensitivity of β-Hexosaminidase, 5'-Nucleotidase, and [^{3}H]-AcNeu7 to Solubilization by 0.03% Digitonin

HeLa cell surface sialic acids were labeled as above in Materials and Methods in Chapter III, and at the stated times were homogenized, and the particulate fraction of the cell-free lysate was treated with 0.03% digitonin as described by Cook et al., (1981). The lysate was then centrifuged in a Beckman Micro Centrifuge for 5 min and the supernatant and pellet assayed for protein, and 5'-nucleotidase, β-hexosaminidase, and radioactivity as described in Materials and Methods in Chapter II. Eight hours after labeling, the cells were then treated with added neuraminidase (N'dase) and resuspended in supplemented S-EMEM.

hours after label		hours after N'dase treatment	% protein recovered	% 5'-Nucleotidase	%[^{3}H]	% β-Hexosaminidase
0	sup	-	32	18	11	72
	pellet	-	68	82	89	28
8	sup	-	15	16	17	70
	pellet	-	85	84	83	30
10	sup	1	22	16	17	68
	pellet	1	78	84	83	32
34	sup	25	27	4	12	69
	pellet	25	73	96	88	31

that after 3 to 5 hours much of the labeled cell surface material had been internalized, the above digitonin data supports the finding that very little of the internalized cell surface is associated with the lysosomal compartment.

The cosedimentation of radiolabeled, surface-derived sialoglycoconjugates with galactosyl transferase activity was investigated by preincubating cells with dextran prior to labeling. Dextran had been previously shown to be internalized into the Golgi of various cell types (Herzog and Farquhar, 1972; Herzog and Reggio, 1980), and it was hoped that such internalization would therefore result in a measurable alteration in the density of Golgi-derived vesicles. The Percoll gradients from control and dextran incubated cells are shown in Figure 8. Plasma membranes band at a density of 1.025-1.035 g/cc in both control and dextran treated cells. In the control cells, galactosyl transferase activity sediments at a density of 1.045-1.055 g/cc, whereas in the dextran-treated cells, the galactosyl transferase sediments at approximately 1.065 g/cc. In addition, the pattern of galactosyl transferase across the entire gradient is altered, whereas substantial galactosyl transferase activity is found in the lighter portion of the control gradient, very little activity is found in the lighter portion of the dextran gradient. In both gradients, β-hexosaminidase sedimented at or near the

Figure 8. Alteration in the density of galactosyl transferase and labeled sialoglycoconjugate containing membranous material by dextran uptake into HeLa cells. A. Radioactivity. B. Galactosyl Transferase Activity.

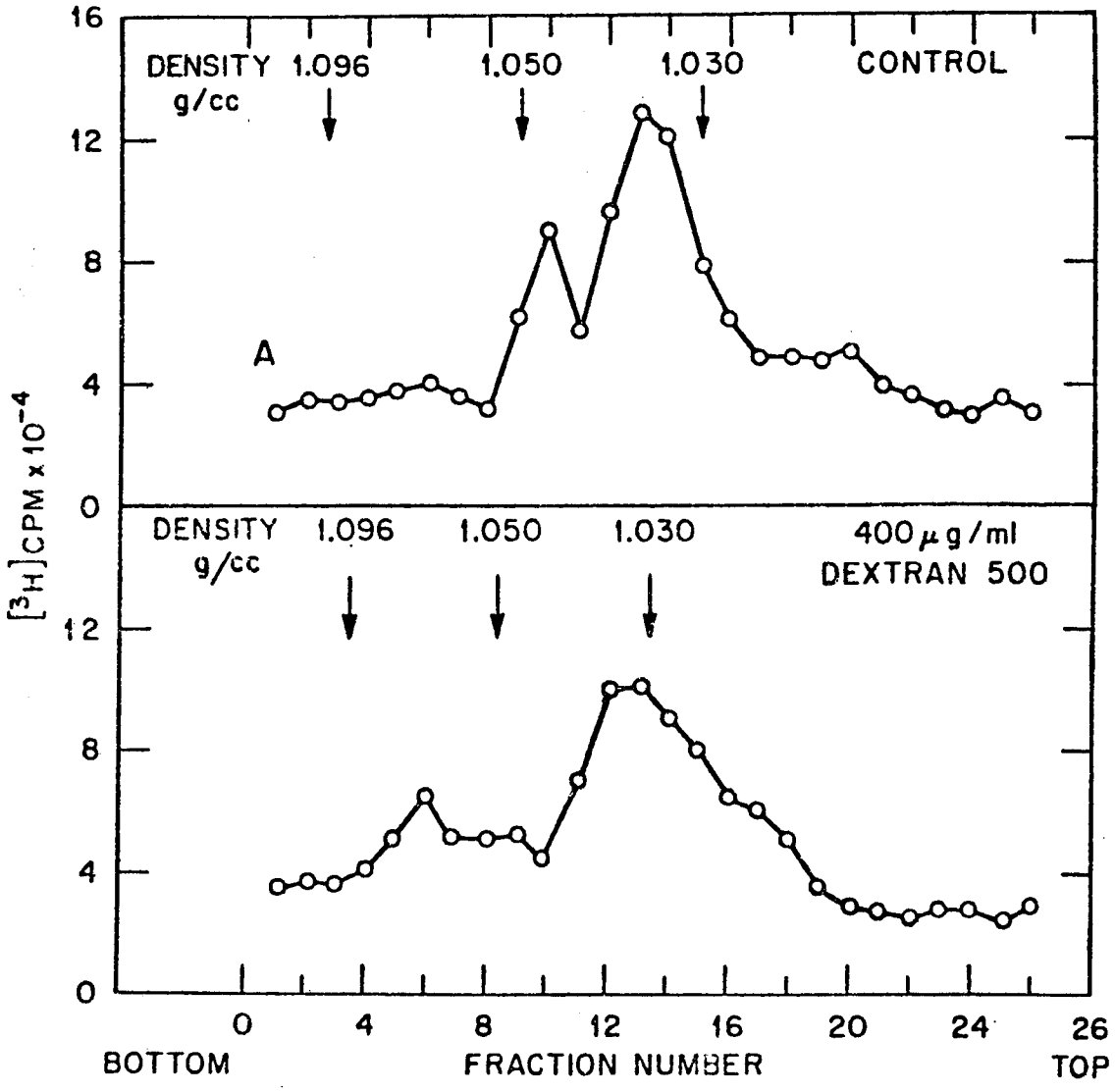

DENSITY g/cc
1.096
1.050
1.030
CONTROL
A
DENSITY g/cc
1.096
1.050
1.030
400 μg/ml DEXTRAN 500
[3H]CPM x 10-4
0
4
8
12
16
20
24
26
BOTTOM
FRACTION NUMBER
TOP

Figure 8

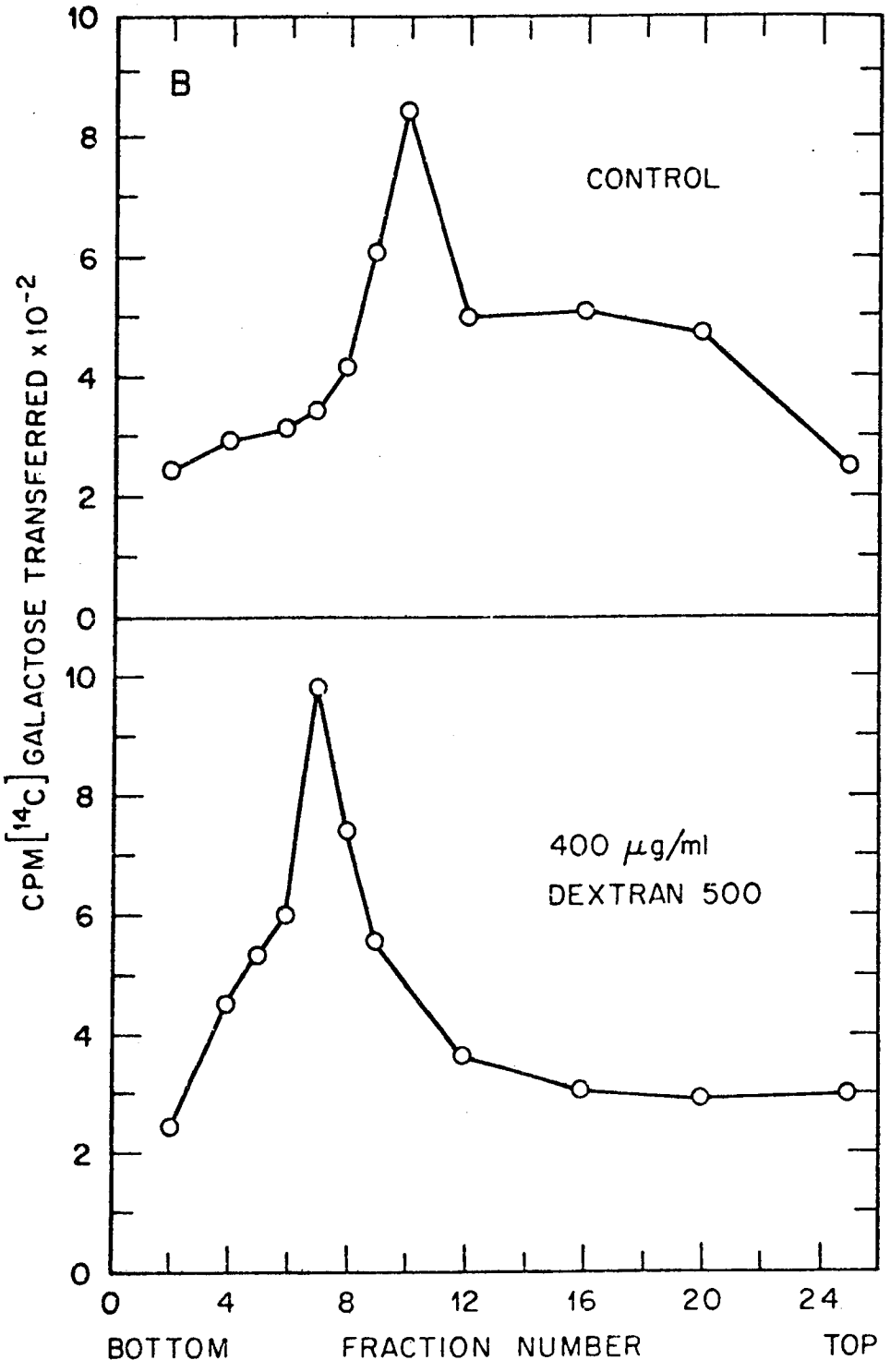


Figure 8, continued

bottom of the gradient (density of 1.09 g/cc or greater). This change in the density of galactosyl transferase-containing vesicles indicated that delivery of dextran from the extracellular medium to the Golgi had taken place.

Temporal Analysis of the Movement of Internalized Sialoglycoconjugates Through Intracellular Organelles

The role of the various organelles in the temporal movement of internalized surface material through the cell was investigated using specialized purification techniques to isolate highly enriched Golgi and lysosomes from HeLa cells. The preparation of the Golgi consisted of three differential centrifugation steps, followed by a four-step sucrose gradient centrifugation. Material from specific interfaces were then pooled and were centrifuged in 17% Percoll-0.25 M sucrose. This final gradient was then fractionated and had the characteristics shown in Figure 9. The galactosyl transferase is concentrated at the light end of the gradient, with its maximum purification exceeding 18-fold over homogenate values.

This fractionation scheme was used to analyze cells at various times after labeling to determine when material from the cell surface becomes associated with the Golgi.

Figure 9. Isolation of enriched Golgi fractions: characteristics of final gradient. Enriched Golgi fractions were isolated as described in Experimental Procedures. The final step in the isolation of enriched Golgi fractions is the centrifugation of material in 17% Percoll at 16,700 RPM in a 60Ti rotor. The 24 ml gradient was fractionated from the bottom into 12-2 ml fractions. The characteristics of this gradient are shown above. The cells had been incubated for 3 hours after labeling.

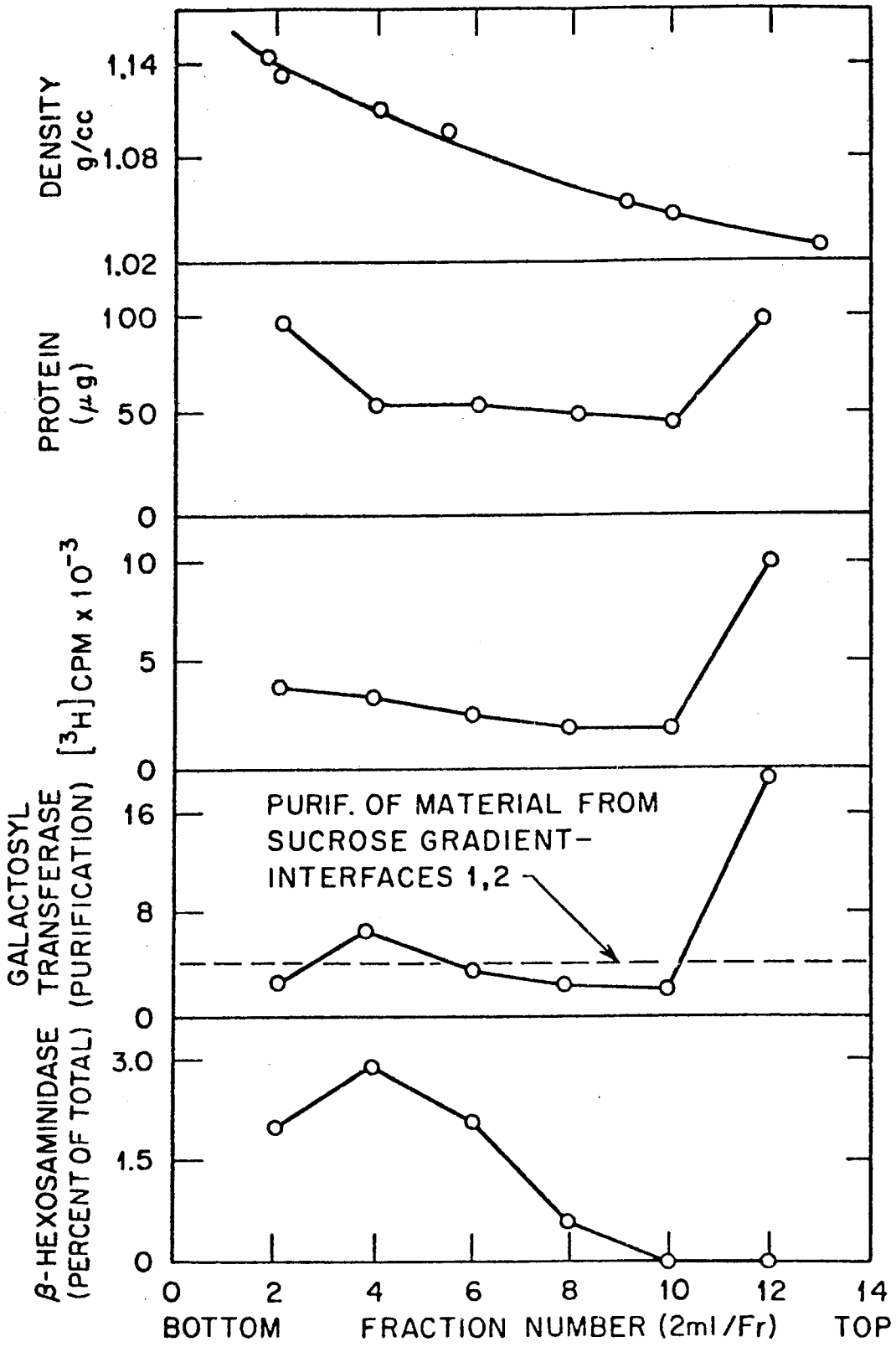

DENSITY g/cc
1.14
1.08
1.02
PROTEIN (μg)
100
50
0
[3H]CPM x 10-3
10
5
0
GALACTOSYL TRANSFERASE (PURIFICATION)
PURIF. OF MATERIAL FROM SUCROSE GRADIENT- INTERFACES 1,2
16
8
0
β-HEXOSAMINIDASE (PERCENT OF TOTAL)
3.0
1.5
0
0
2
4
6
8
10
12
14
BOTTOM
FRACTION NUMBER (2ml/Fr)
TOP

Figure 9

Figure 10 illustrates the movement of material into the galactosyl transferase enriched fractions 1, 3, 8, and 15 h after labeling. During the first hour after labeling, only a small amount of radioactive material is seen moving into the Golgi. Between 1 and 3 hours after labeling, however, there is a large increase in the radioactivity seen associated with the Golgi. From 3 to 8 h, there is no signifcant change in the specific raidoactivity of the low density fractions. A change is observed between 3 and 8 h, however, in the more dense fractions (approximately 1.07-1.08 g/cc) which contains both -hexosaminidase and galactosyl transferase activity and may, in fact, be related to the GERL (Golgi-endoplasmic reticulum-lysosomes). This presumptive GERL is loaded with cell surface-derived sialoglycoconjugates between 3 and 8 hours after labeling. There is no change observed in this relative pattern between 8 and 15 h after labeling.

Highly enriched preparations of lysosomes could be isolated using a four-step gradient to separate fractions from labeled cells exposed to dextran as above. This procedure, was a modification of the procedure of Thines-Sempoux as described (Beaufay, 1972), and allowed the isolation of lysosomes enriched approximately 22 fold over homogenate. As illustrated in Figure 11, the specific radioactivity of the enriched lysosomal fraction reached a

Figure 10. Temporal movement of cell surface derived material into enriched Golgi fractions. The isolation procedure described in the legend to Figure 9 was used to analyze the movement of cell surface material into Golgi enriched fractions versus time. The symbols are: ○, 1 h; ●, 3 h; △, 8 h; ▼, and 15 h after labeling.

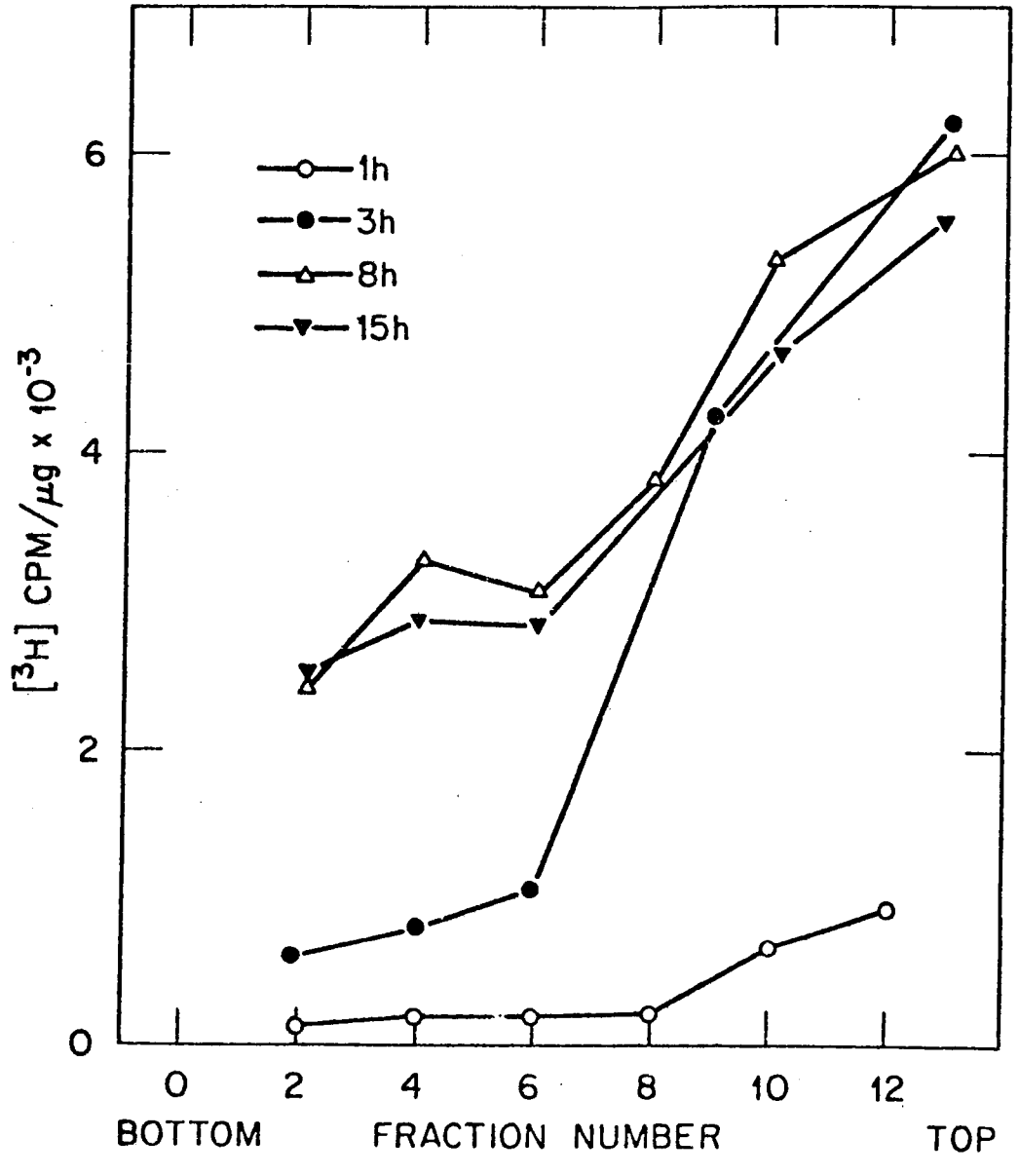

1h
3h
8h
15h
[3H] CPM/μg x 10-3
0
2
4
6
0
2
4
6
8
10
12
BOTTOM
FRACTION NUMBER
TOP

Figure 10

Figure 11. Isolation of purified lysosomes. Purified lysosomes were isolated by either the dextran isolation method of Thines-Sempoux as described (Beaufay, 1972) or by the detergent method of Trouet (1964), as described in Materials and Methods. HeLa cells were incubated in the presence of either dextran 500 (0.4 mg/ml) for 3 h or 0.02 mg/ml Triton WR-1339 for 24 h prior to labeling of the cell surface. At the times indicated, lysosomes were isolated and assayed for protein, radioactivity, and β-hexosaminidase activity.

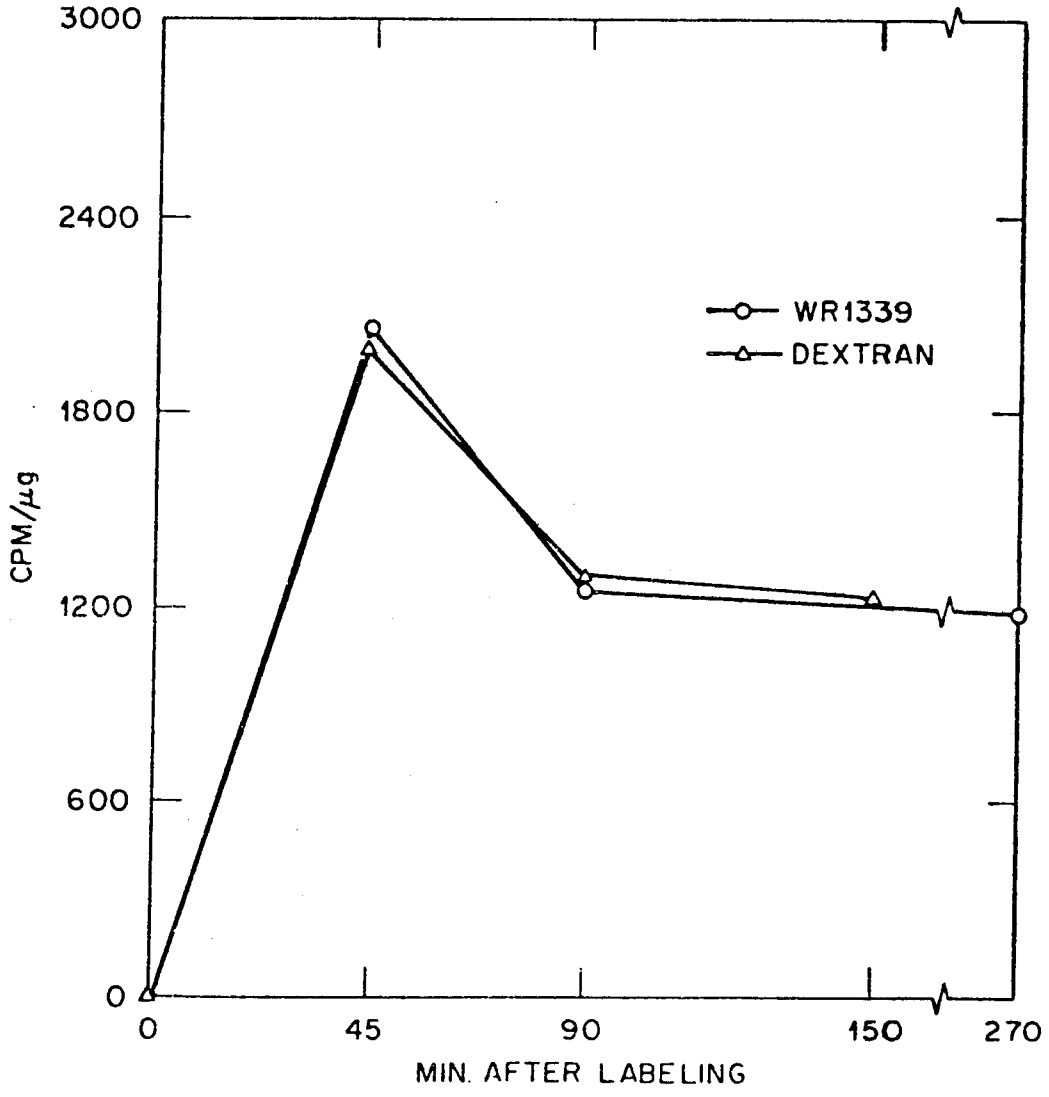

3000
2400
1800
1200
600
0
CPM/μg
WR1339
DEXTRAN
0
45
90
150
270
MIN. AFTER LABELING

Figure 11

peak of associated radioactivity approximately 30-60 min after labeling, declining by about one-third by 90 min, with a slower decline after 90 min. A second purification scheme, based on the uptake of the nonionic detergent, Triton WR-1339 into the lysosomes to decrease their density (Trouet, 1964), yielded similar results, also shown in Figure 11.

Effects of NH_4Cl or Chloroquine on the Redistribution of Internalized Cell Surface Sialoglycoconjugates

The differing rates of uptake of internalized cell surface material into the lysosomal and Golgi compartments shown in Figures 10 and 11 suggest that the endocytosed material is first associated with the lysosomes and then makes its way, presumably via vesicular transport, to the Golgi complex. Further substantiation for such a pathway comes from experiments using NH_4Cl or chloroquine, agents which disrupt the normal functioning of the lysosomes by raising the intralysosomal pH (DeDuve et al., 1974; Ohkuma and Poole, 1978; Christopher and Morgan, 1981). The change in intralysosomal pH may among other things impair the function of degradative enzymes found in the lysosomes, increasing the turnover times for cell surface constituents and/or interfere with the intracellular transfer of internalized, cell surface material between

various cellular organelles, both of which may affect the rate and extent of recycling.

Dextran, coupled to fluorescein (FD) was used to estimate the pH of the endocytic vesicles and lysosomes (Figure 12). When cells were incubated with FD for ten minutes, washed, and resuspended at 18°C in medium without FD there is a drop in the pH of the environment containing FD to about pH 5.8-6.0 in three to five minutes. The incubation of cells at 18°C has been previously shown (Dunn et al., 1980; Weigel and Oka, 1981; Wolkoff et al., 1984) to inhibit the fusion of endocytic vesicles with the lysosomes, but does not seem to significantly interfere with the internalization or recycling of the asialoglycoprotein receptor (Wolkoff et al., 1984). It was therefore concluded that within 3 to 5 minutes after the uptake of FD, this material is sequestered in an acidic compartment distinct from the lysosomes. If the cells are then warmed to 37°C, there is a further drop in the pH within 10-15 minutes to approximately pH 5.0, thought to be due to the fusion of endocytic vesicles with lysosomal elements. If NH_4Cl is then added to the extracellular medium at a concentration of 2 mM, there is an almost immediate rise in the pH of the environment of the FD to approximately pH 6.5-7.0. This effect has been previously demonstrated by Ohkuma and Poole (1978) to be

Figure 12. Estimation of the pH within endocytic vesicles and lysosomes. HeLa cells, allowed to attach to uniform pieces cut from microscope slides, were incubated at 18°C for 10 min in the presence of fluorescein conjugated to dextran (FD) of molecular weight 70,000. The cells were then washed and resuspended at 18°C to obtain an estimate of the pH of endocytic vesicles. The cells were then incubated at 37°C which allowed enbdocytic vesicles to fuse with lysosomes, thus enabling an estimation of the pH of the lysosomes. Fluorescence was measured at 519 nm with excitation at 450 and 495 nm. The ratio of the fluorescence at 495 to that at 450 was converted to pH using a standard curve (inset) of FD in various buffers from pH 4.5 to 7.5.

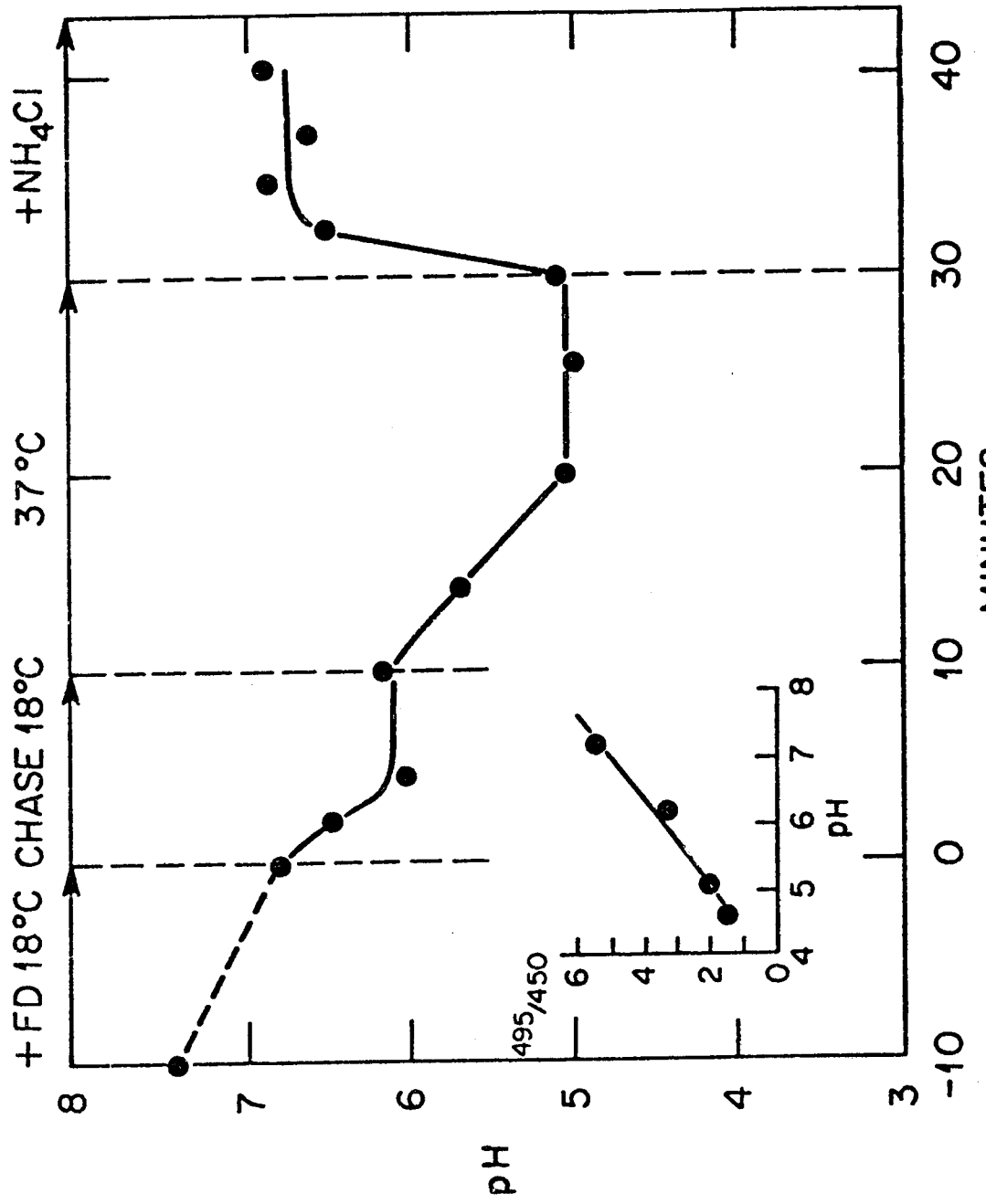

+FD 18°C
CHASE 18°C
37°C
+NH4Cl
pH
MINUTES
8
7
6
5
4
3
-10
0
10
20
30
40
495/450
6
4
2
0
4
5
6
7
8
pH

Figure 12

due to dissipation of the pH gradient of the lysosomal compartment. Percoll gradient density centrifugational Percoll gradient analysis was then performed on cells treated with NH_4Cl or chloroquine, to determine if the dissipation of the pH gradient of the lysosomal compartment would interfere in the transfer of material from the lysosomes to the Golgi.

As seen in Figure 13, the addition of either NH_4Cl or chloroquine to HeLa cells immediately after labeling results in a marked increase in the specific radioactivity of lysosomes isolated 45, 90, and 150 minutes after cell labeling, as compared to control cultures. The impairment of intralysosomal protein degradation could not account for the large difference that was observed in the specific radioactivity of lysosomes isolated from the control or the treated (NH_4Cl or chloroquine) cells due to the slow (approximately 2% per h) rate of degradation of surface proteins in untreated cells as described in Chapter Two.

It is, however, possible that these agents caused an overaccumulation in the lysosomes of material that may otherwise be transferred to other intracellular organelles and/or recycle back to the cell surface. To determine the effects of these agents on intracellular movement of material in HeLa cells, Percoll gradient analysis (see Figure 7 and Materials and Methods for details) was used.

Figure 13. Overaccumulation of cell surface material within lysosomes upon the addition of NH_4Cl or chloroquine. Lysosomes were isolated using the dextran loading method described in Materials and Methods. Labeled HeLa cells were incubated for the times indicated in the presence of both dextran and either NH_4Cl or chloroquine, the cells harvested, and the specific radioactivity and purification of the lysosomes assessed.

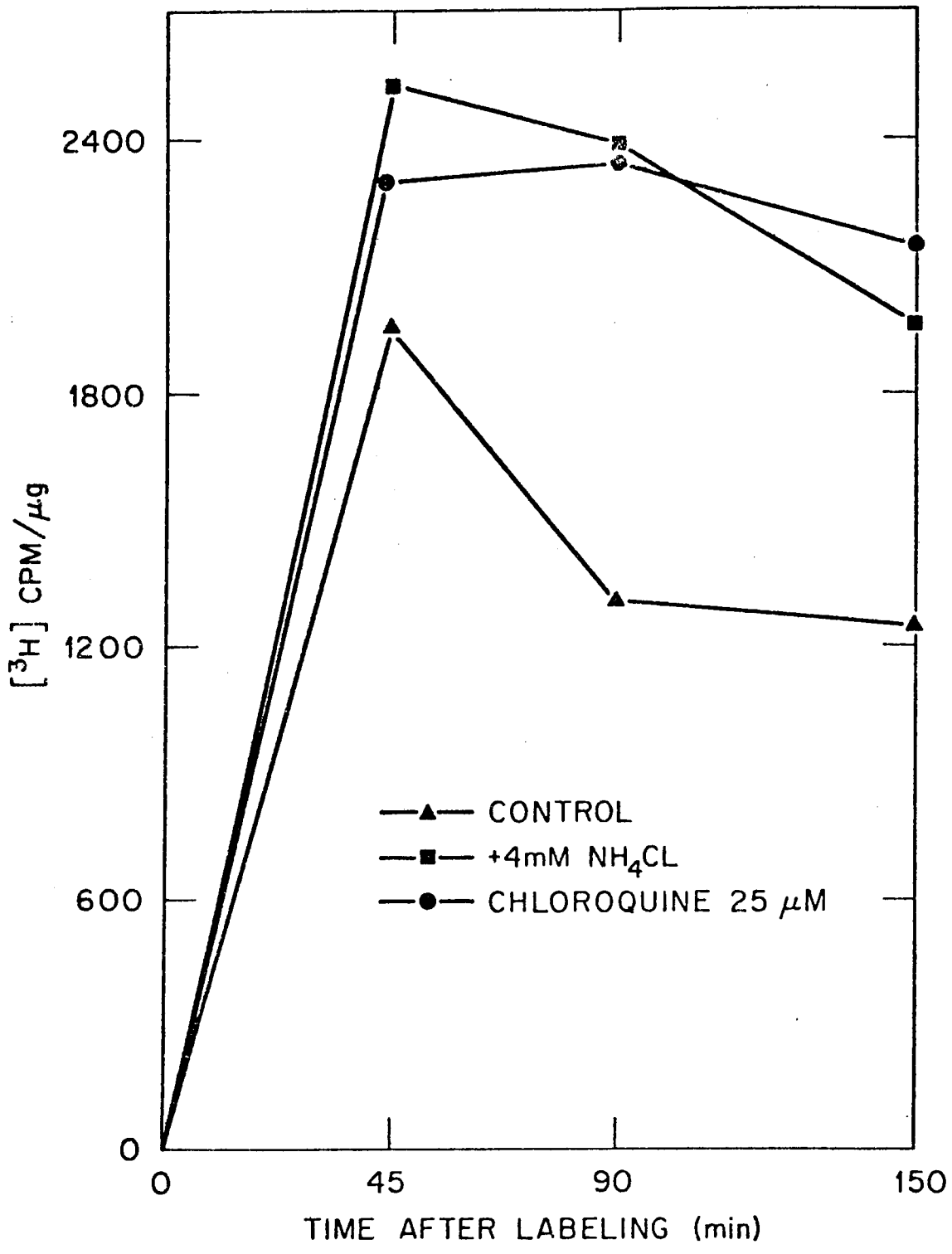

2400
1800
1200
600
0
[3H] CPM/μg
0
45
90
150
TIME AFTER LABELING (min)
CONTROL
+4mM NH4CL
CHLOROQUINE 25 μM

Figure 13

Labeled cells, incubated in control medium or with NH_4Cl or chloroquine, were homogenized and the post-nuclear supernatant added to 22.5% Percoll-0.25 M sucrose, 10 mM Tris and centrifuged. Figure 14 illustrates the results from these gradients, revealing that at 150 min in control cultures only, a large peak of radioactivity cosediments with the Golgi marker, galactosyl transferase. In the cultures incubated in the presence of NH_4Cl or chloroquine, decreased levels of radioactivity were seen to cosediment with galactosyl transferase. Unlike that seen in control cultures, however, galactosyl transferase in cells incubated in the presence of dextran and either chloroquine or NH_4Cl sedimented at a density of around 1.050-1.055 g/cc, resembling more the density for the Golgi seen in non-dextran incubated cultures. Thus, the inhibition of transfer of radioactive material from the plasma membrane to the Golgi by NH_4Cl or chloroquine also inhibited to some extent the transfer of dextran from the extracellular fluid to the Golgi.

The effect of chloroquine on recycling in HeLa cells is shown in Figure 15. Cells were labeled, incubated at 37°C for 8 hours in S-EMEM, during which time substantial internalization of the cell surface occurs, and then were treated with neuraminidase to remove remaining surface radioactivity. The cells were reincubated for the times shown and a second neuraminidase treatment performed.

Figure 14. Lack of cell surface material accumulating within the Golgi of labeled HeLa cells in the presence of chloroquine or NH_4Cl. Labeled HeLa cells were analyzed by Percoll gradient centrifugation as described in Materials and Methods above after incubation in the presence of chloroquine (□), NH_4Cl (Δ), or control medium only (○).

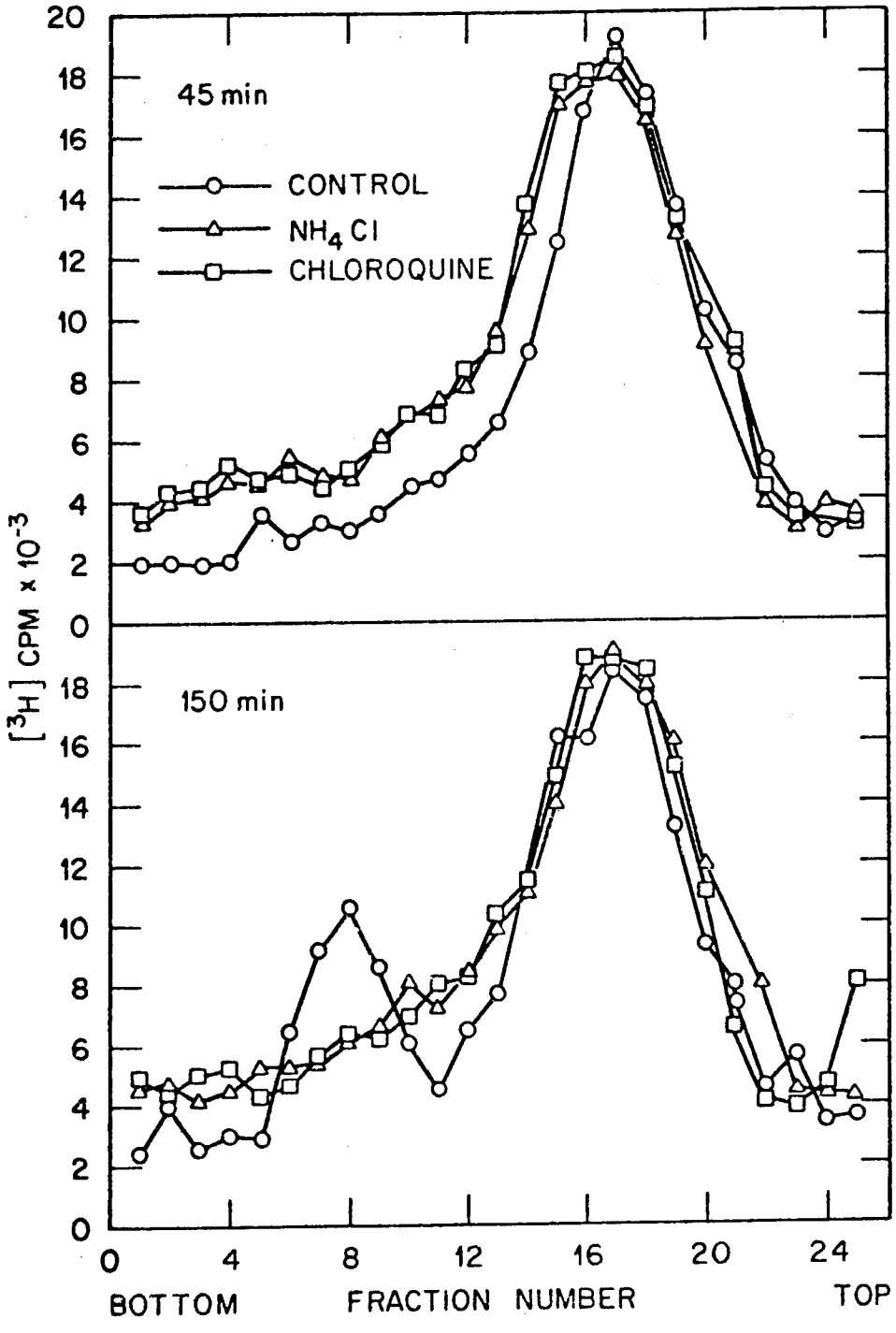

45 min
CONTROL
NH4 Cl
CHLOROQUINE
150 min
[3H] CPM x 10-3
FRACTION NUMBER
BOTTOM
TOP

Figure 14

Figure 15. Recycling of internalized cell surface material is inhibited by chloroquine in HeLa cells. HeLa cells, labeled as described above, were incubated for 15 h to allow internalization of the cell surface. The cells were then exposed to neuraminidase(N'dase I) as previously described (5) to remove the remaining cell surface radioactivity. The cells were then reincubated for the times indicated and a second neuraminidase treatment (N'dase II) performed to assess the amount of the above radioactivity that had returned to the surface. Cells were either incubated in control medium (□), in the presence of 100 uM chloroquine during the neuraminidase treatment (Δ) and after, or in the presence of 100uM chloroquine after neuraminidase had been removed (O).

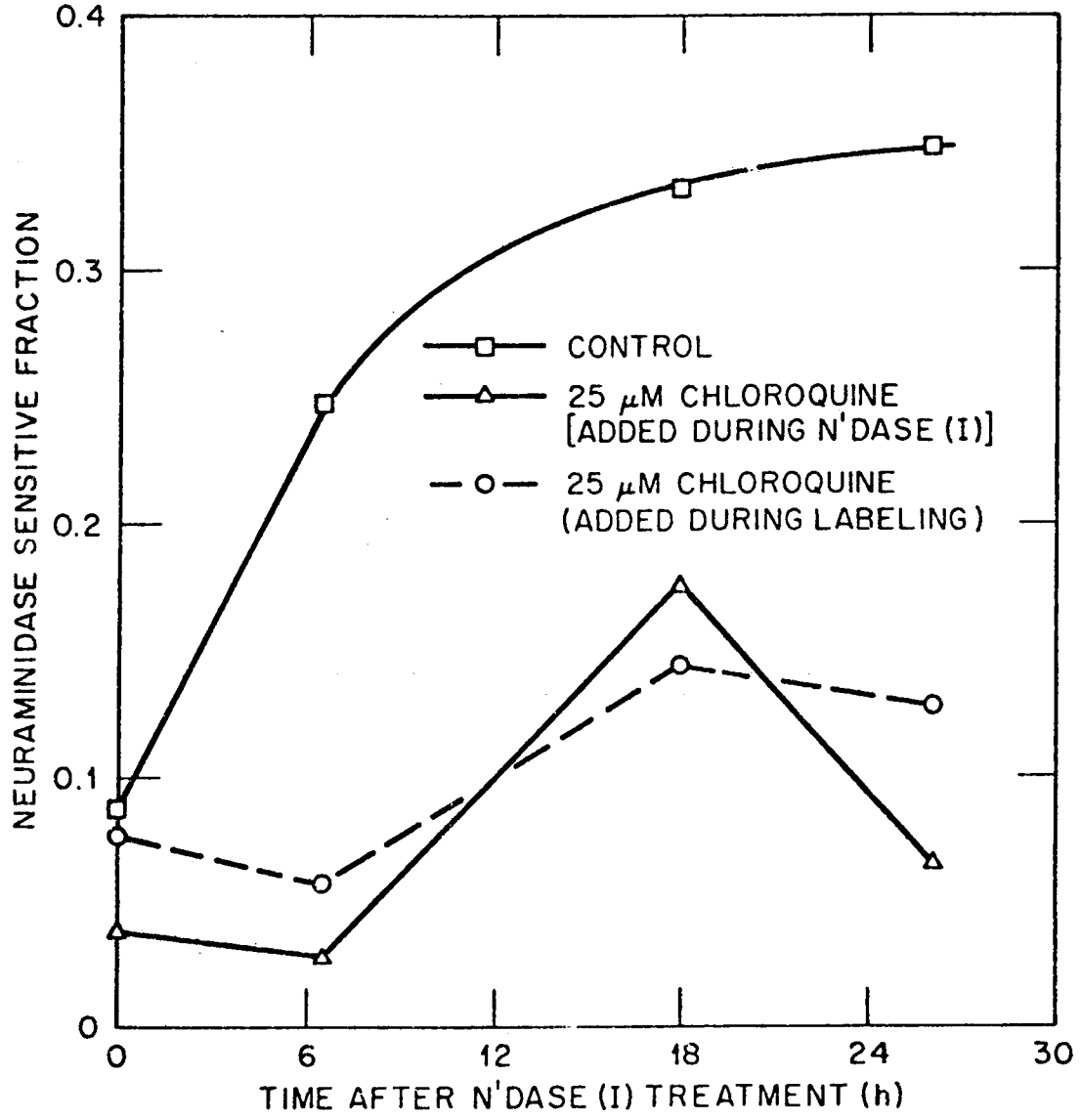

0.4
0.3
0.2
0.1
0
NEURAMINIDASE SENSITIVE FRACTION
CONTROL
25 μM CHLOROQUINE
[ADDED DURING N'DASE (I)]
25 μM CHLOROQUINE
(ADDED DURING LABELING)
0
6
12
18
24
30
TIME AFTER N'DASE (I) TREATMENT (h)

Figure 15

As shown, chloroquine significantly inhibited the return of the internalized, cell surface sialoglycoconjugates (recycling) to the cell surface. It should be noted that the neuraminidase treatment(s) did not have any effect on intracellularly located radioactivity (Figure 17 and data not shown). Taken together with the increased labeling of the isolated lysosomes in the presence of either NH_4Cl or chloroquine, it seems as though these agents disrupt the flow of membranous material by causing an accumulation of internalized, surface material primarily in the lysosomal compartment.

Discussion

The data presented in this chapter demonstrate that internalized, cell surface sialoglycoconjugates can be transferred intracellularly from endocytic vesicles to the lysosomal compartment, and may then undergo transfer to the Golgi apparatus. There is also some evidence for the existence of a third compartment, slightly more dense than the Golgi and possibly related to the GERL, which is loaded with internalized surface material temporally after the Golgi, but is not loaded if the transfer of material from the lysosomes to the Golgi has been inhibited.

Establishing the Golgi as the Major Storage Site for Internalized, Cell Surface Sialoglycoconjugates

No consensus has been reached as to the role of the Golgi in the processes of endocytosis and recycling. The EM visualization of cationic ferritin (CF) labeling has produced inconsistent results as to the movement of internalized surface material into the Golgi and/or the GERL (Farquhar, 1978; Herzog and Miller, 1979; Thyberg and Stenseth, 1981; Tartakoff et al, 1981; Van Deurs et al., 1981; Van Deurs et al., 1982). I therefore felt it was necessary to utilize several different fractionation procedures to determine if there was an association of internalized surface sialoglycoconjugates with the Golgi complex. In addition, a high molecular weight dextran, previously shown to be internalized into the Golgi in some cell types (Herzog and Farquhar, 1977; Herzog and Reggio, 1980), was used to further substantiate the association of surface-derived material by altering the density of the Golgi vesicles and the surface-derived radioactivity. The various methods of Golgi isolation yielded similar results as to the association of internalized surface material with the Golgi. In addition, similar time courses for uptake of this material into the isolated Golgi fractions were obtained. In control cultures (1-3 h after labeling and beyond), galactosyl transferase and a peak of internal

tritium radiolabeled, cell surface sialoglycoconjugates were observed to cosediment at a density of approximately 1.05-1.055 g/cc. When cells are incubated in the presence of dextran for at least 12 hours, and the cells labeled and reincubated at 37°C for 3 hours, both the peak of galactosyl transferase and the peak of radioactivity which normally sedimented at a density of 1.05 g/cc now sedimented at a density of 1.065 g/cc. It was therefore concluded that the cell surface sialoglycoconjugates were being internalized into vesicles containing galactosyl transferase activity. Since this enzyme has been shown to be an unequivocal marker for the Golgi complex in HeLa cells (Strous and Berger, 1982), it thus follows that the internalized surface components could reach the Golgi complex in HeLa cells.

Temporal Analysis of the Intracellular Movement of Internalized Surface Material

The association of internalized, cell surface membrane constituents with the lysosomal compartment is well documented in a variety of cellular systems (Muller et al., 1980; Van Deurs and Nilausen, 1982). It was still unclear, however, what role the lysosomes may play in the complex flow of membrane during endocytosis and recycling. Two pieces of evidence are presented in favor

of movement of material from the plasma membrane to the lysosomes in HeLa cells, and then to the Golgi complex. The time course of uptake of internalized cell surface material into the lysosomes and the Golgi was markedly different, with the lysosomes reaching a peak of associated cell surface radioactivity approximately 45 minutes after labeling and the Golgi reaching a peak at approximately three hours after surface labeling. Secondly, in the presence of agents which raise the intralysosomal pH, NH_4CL or chloroquine (DeDuve et al., 1974; Ohkuma and Poole, 1978; Christopher and Morgan, 1981), there was an accumulation of radiolabeled material in the lysosomes and an inhibition of cell surface recycling, with very little radiolabeled material seen associated with the Golgi. This strongly suggests that the majority of internalized surface material which becomes associated with the Golgi complex in HeLa cells passes first through the lysosomes. In addition, some data was presented which suggested the existence of a more dense compartment which contains both -hexosaminidase and galactosyl transferase activities. Internalized surface membrane becomes associated with this compartment 3 to 8 hours after labeling. In addition, if NH_4Cl or chloroquine were present, interfering with the loading of surface material into the Golgi, no loading of the above compartment occurred. The nature of the enzyme

activities associated with this unknown compartment as well as its density suggest that it may be related to the GERL (Golgi-endoplasmic reticulum-lysosomes; Novikoff and Novikoff, 1977). The similarity in the time course for the introduction of internalized, surface-derived material into the presumptive GERL (between 3 and 8 hours after labeling) with the half-time for recycling of 4-6 hours suggests that this presumptive GERL may be involved in recycling. Previous studies reported that the movement of lectins and other PM ligands into the GERL (Joseph et al., 1979; Thyberg, 1980) suggesting that the extracellular material and also perhaps the plasma membrane can become associated with the GERL.

The conflicting reports in the literature as to the transfer of material from the cell surface to the Golgi (Farquhar, 1978; Herzog and Miller, 1979; Thyberg and Stenseth, 1981; Van Deurs et al., 1981; Van Deurs et al., 1982) suggest that there may be number of pathways available for the processing of internalized material, and that a cell's function could determine predominance of one or another pathway. Farquhar and coworkers (Wilson et al., 1981), using the IgG-secreting murine myeloma cell, demonstrated that [^{125}I]-labeled cell surface components as well as cationic ferritin become associated with the Golgi complex stacks. In addition, less than 1% of the

cell-associated radioactivity was seen associated with lysosomal elements. This compares to my measurement of 10 to 20% of the cell-associated radioactivity being lysosomally associated and up to 50% or more of the cell-associated CF in other studies (Van Deurs and Nilausen, 1982) being found associated with the lysosomes. In studies where large labeling of the lysosomes occurs, there is a curious lack of Golgi complex labeling. This suggests that several pathways for the processing of the internalized surface membrane may be operating. The first pathway predominates in secretory and highly endocytosing cells, resulting in delivery of endocytic vesicles and their contents directly to the Golgi. This would allow for a more rapid reutilization of cell surface components in cells undergoing extensive endo- and exocytosis. A second pathway may predominate in HeLa cells, transfering material first to to the lysosomes and then to the Golgi complex. The third possible pathway may involve direct delivery of surface material to the lysosomes with only low levels of this surface-derived material reaching the Golgi complex.

In secreting cells, CF labeling of the Golgi complex is observed, with very little, if any labeling of the lysosomes. This suggests that surface membrane is also moving directly to the Golgi, the site of secretion

granule synthesis for reutilization, thus allowing secretion to proceed rapidly, and without the loss of surface material via lysosomal degradation. The second pathway could have resulted in the CF being sequestered intralysosomally at first, with slower transfer then occurring to the Golgi, as is observed here for HeLa cells. In cell types where the rate of internalization of the cell surface is very low, the requirement for any internalized surface material movement may also be low, thus resulting in most of the internalized, cell surface material associating with the lysosomes (Van Deurs and Nilausen, 1982).

In HeLa cells, the data presented here indicate the movement of surface material occurs first to the lysosomal compartment, and from there to the Golgi complex. The route of movement from the Golgi to the above mentioned presumptive GERL is unclear. The loading of this latter compartment clearly takes place between three and eight hours after labeling, suggesting transfer from the Golgi, which is loaded between 1 and 3 h with surface-derived material. Other routes for the introduction of surface-derived material have not been ruled out, however.

It has been reported by Basu et al. (1981), that chloroquine was only a weak inhibitor of LDL receptor recycling in human fibroblasts. This contrasted with

their finding of an almost complete inhibition of recycling upon the addition of the monovalent carboxylic ionophores monensin and nigericin. These ionophores catalyze the exchange of a proton for sodium and for potassium, respectively (Pressman, 1976), produce gross morpholigical alterations of the Golgi complex (Ledger et al., 1980), and have been reported to raise the lysosomal pH (Ohkuma and Poole, 1978). The ionophores were thought to inhibit recycling of the LDL receptor through this intralysosomal pH elevation, though the difference in the inhibition of recycling by chloroquine and monensin could suggest otherwise, since chloroquine causes a rapid and also reversible dissipation of the lysosomal pH gradient (Ohkuma and Poole, 1978). Basu et al. (1981) have also reported that during this inhibiton of the recycling by monensin, large numbers of perinuclear vacuoles distinct from lysosomes were seen to be the site of LDL receptor location intracellularly. The lack of inhibition of recycling by chloroquine may, therefore, be due to the majority of the internalized, plasma membrane material associating not with the lysosomal compartment, but with the Golgi complex. Monensin would be a much better inhibitor of LDL receptor recycling due to its effects on the Golgi complex, whereas chloroquine may not effect receptor systems which utilize primarily the plasma membrane to

Golgi to plasma membrane route of movement. In the case of the transferrin receptor (Ciechanover et al., 1983) and asialoglycoprotein receptor (Schwartz et al., 1984) which deliver their ligands to the lysosomal compartment, amines effectively inhibit receptor recycling. Sensitivity to these various agents may therefore allow for determination of the pathway involved in the intracellular disposition of internalized receptor systems.

It would be interesting to determine if the route of movement of surface derived material could be altered by modulating a cell between a secreting form (high rate of exo- and endocytosis) and a non-secreting form. Whether certain cell types are predisposed towards predominance of one or another processing pathways or whether the state of cell determines pathway predominance could be studied. Cellular differentiation could result in a non-secreting cell, which may use predominantly a lysosomally-dependent processing pathway, differentiating into a secreting cell, using predominantly the direct plasma membrane to Golgi route of endocytic vesicle movement to speed up recycling and decrease the rate of degradation via a decrease in the amount of material associated with lysosomal compartment.

In conclusion, internalized cell surface sialoglycoconjugates can become associated with the lysosomal compartment in HeLa cells, from which they can undergo

transfer to the Golgi, and then, perhaps, to a more dense compartment which may be the GERL. The halftimes for recycling and the loading of the presumptive GERL are very similar, suggesting that recycling may ultimately occur from this compartment, as was suggested by previous work (Palade, 1975; Meldolesi et al., 1978; Whaley and Dauwalden, 1979).

CHAPTER IV

SORTING OF INTERNALIZED CELL SURFACE SIALOGLYCOPROTEINS

Introduction

The plasma membrane is a dynamic structure, and its components are contantly being degraded and synthesized. Since the turnover of the components may vary depending upon the physiological state of the cell due to up- or down-regulation or due to alterations in gene expression, it is probable that not all cell surface components are undergoing internalization and/or recycling with the same kinetics (see Chapter I for details). Thus, sorting of cell surface components must take place at many levels of cellular organization to ensure proper composition of the plasma membrane. The cell surface components undergoing internalization must be separated from the components not being internalized at the level of the cell surface. A more complicated type of sorting must take place when several cell surface components are internalized, some of which will undergo recycling and some of which will not. These components must be separated from each other at some intracellular site or sites. The site(s) where this type

of sorting takes place has not yet been identified. It was recently postulated by Wolkoff et al. (1984) that for the asialoglycoprotein receptor, this separation comes at the level of the endocytic vesicle and/or the endosome. Though endocytic vesicles fuse to become endosomes, the temporal relationship between the endocytic vesicles, endosomes, receptosomes, and sorting has not been worked out (Pastan and Willingham, 1981; Wolkoff et al., 1984). The asialoglycoprotein receptor releases its ligand probably through acidifcation of the endocytic vesicle and cytoskeleton-dependent segregation (Wolkoff et al., 1984) and can then quickly recycle (t/2=7-15 min; Schwartz et al., 1984). There is no evidence at this time that the asialoglycoprotein receptor associates with any other intracellular organelles except the endocytic vesicles during its recycling. This rapid recycling was also observed for the transferrin receptor ($\cong$21 min; Bleil and Bretscher, 1982) and may in fact be a characteristic of all ligand-receptor internalization via coated pits and vesicles. The situation discussed in Chapters II and III suggests that sorting may also take place at the level of other cellular organelles (e.g., lysosomes, Golgi). The movement of material through the lysosomal compartment may itself be a form of sorting, via the degradation of certain cell surface components, while others are not

degraded and are transfered to another cellular organelle. This inter-organelle movement of internalized surface material undoubtedly results in the longer recycling times observed for sialoglycoproteins reported in Chapter III.

Since the labeling technique used here to study recycling labels the cell surface directly, the surface derived components associated with the various subcellular fractions described in Chapter III could be determined. The fluorographic analysis of the electropherograms from these fractions allowed for the identification of several sorting sites for internalized cell surface material in HeLa cells as summarized below.

Materials and Methods

The isolation of plasma membranes, labeling of cell surface sialoglycoconjugates, and the assays for protein, -hexosaminidase, and galactosyl transferase activity were all described in Materials and Methods in Chapters II and III.

Gradient Gel Electrophoresis

SDS-PAGE was performed as described in Chapter Two, with the exception that 4-10% gradient gels were used. These were run on an apparatus supplied by LKB instruments.

Gels were impregnated with PPO and DMSO and exposed to Kodak XAR film that had been preflashed as described (Laskey and Mills, 1976).

Densitometric Scanning of Fluorograms

The density pattern of the fluorographs of dried SDS-polyacrylamide gradient gels were scanned using an LKB UltroScan Densitometer, Model 2022, interfaced to an Apple IIe, and kindly provided by Dr. Frank Kenney. Backround determinations and integration of peaks were performed using software (GELSCAN) supplied by LKB. Curves were fit by the computer to correspond to Gaussian fits with better than 5% accuracy.

Percoll Gradient Analysis

Percoll gradients were run as was described in Chapter III with some minor modifications. The gradients were fractionated and the fractions for gel analysis in the appropriate figures were centrifuged for 3 h at 40,000 RPM in a 50Ti rotor to remove Percoll. The Percoll formed a hard pellet and the supernatant, containing the material to be assayed, was removed immediately and then stored at -70^{o}C. Samples of these fractions were assayed for the appropriate markers and another sample was diluted 1:3 with gel solvent. The gel samples were heated to 100^{o}C

for 2 min and 0.1 ml of each sample was applied to the gradient gels which were run as described above.

Electrophoretic Analysis of Endosomes and Endocytic Vesicles

Experiments designed to analyze the internalization of cell surface sialoglycoproteins into endosomes and/or endocytic vesicles were performed at 18°C, which has been shown to prevent fusion of endocytic vesicles with the lysosomal compartment (Dunn et al., 1980; Weigel and Oka, 1981; Wolkoff et al., 1984). Cells were labeled as was described in Chapter III, and were resuspended for 60 min at 18°C. The cells were then exposed to neuraminidase (2 units/ml) at pH 5.0 for 120 min at 18°C, or incubated under the same conditions without the neuraminidase for control gradients. The cells were then washed twice in cold PBS and Percoll gradient analysis was performed as described in Chapter III. Gradient fractions that corresponded to the density of the endocytic vesicles and/or the endosomes were centrifuged as above to remove Percoll and then analyzed via gradient gel electrophoresis followed by fluorography.

Results

HeLa cell surface sialoglycoproteins can be labeled via sequential treatment of the cells at 0 to 4°C with $NaBH_4/NaIO_4/NaB[^3H]_4$. When plasma membranes from labeled cells are analyzed by SDS-PAGE followed by fluorography as described above (Figure 16, lane 1), a large number of labeled protein bands are observed (approximately 16). Quantitation of the radioactivity present in each band is possible by preflashing film prior to autoradiographic exposure, as described by Laskey and Mills (1976), which results in a linear relationship between density of x-ray film and radioactivity. This chapter will concern itself with the fate of seven major radioactive bands, indicated by the arrowheads seen in Figures 16 and 17, whose nominal molecular weights are 210K, 180K, 135K, 105K, 96K, 63K and 45K, and will be referred to as bands 1 through 7, respectively.

Visualization of Cell Surface Sialoglycoprotein Recycling

If any cell surface sialoglycoproteins were being preferentially sorted at the level of the cell surface or intracellularly, leading to preferential recycling or degradation, then the SDS-PAGE pattern of radiolabeled sialoglycoproteins on the cell surface should change with time. To test whether this was occurring, cells were

Figure 16. SDS-PAGE fluorographic pattern of labeled surface sialoglycoproteins with time. HeLa cell surface sialic acids were labeled as in Materials and Methods in Chapter Three, and plasma membranes isolated at the times indicated. The arrowheads indicate the major bands. The gel was a 4-15% acrylamide gradient, with each lane loaded with equal radioactivity. The gel was impregnated with PPO, dried down, and incubated at -70°C for five days with Kodak XAR film as described in Materials and Methods.

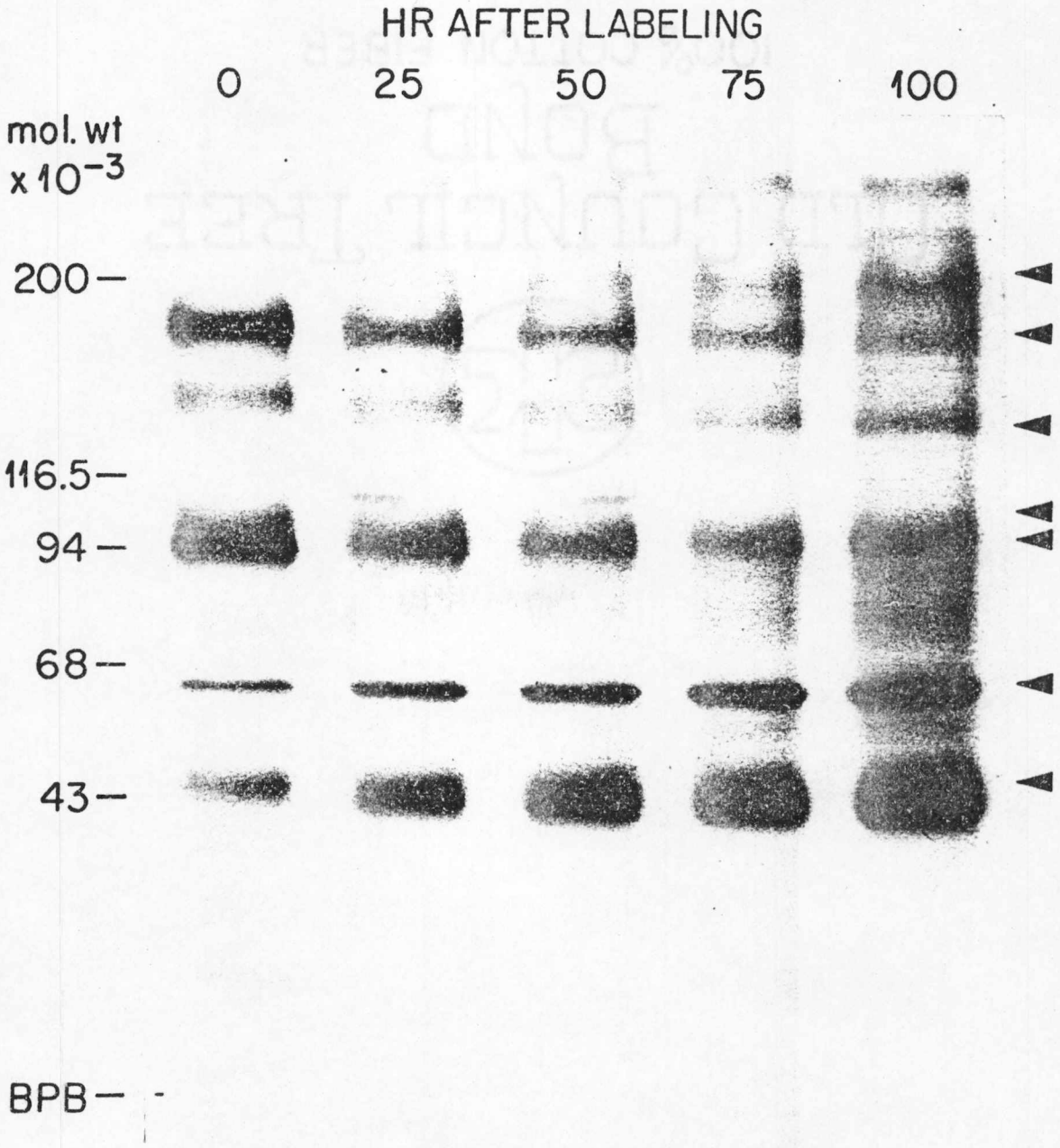

HR AFTER LABELING
0
25
50
75
100
mol. wt
x 10⁻³
200
116.5
94
68
43
BPB

Figure 16

Figure 17. Fluorographic visualization of recycled cell surface sialoglycoproteins. SDS-PAGE was used to analyze plasma membranes (PM; lanes 1-3) isolated immediately after labeling, immediately after neuraminidase treatment, and 8 h after neuraminidase treatment. In addition, the total particulate fraction was isolated immediately after neuraminidase treatment. The methods were described in the legend to Figure 16 and in Materials and Methods.

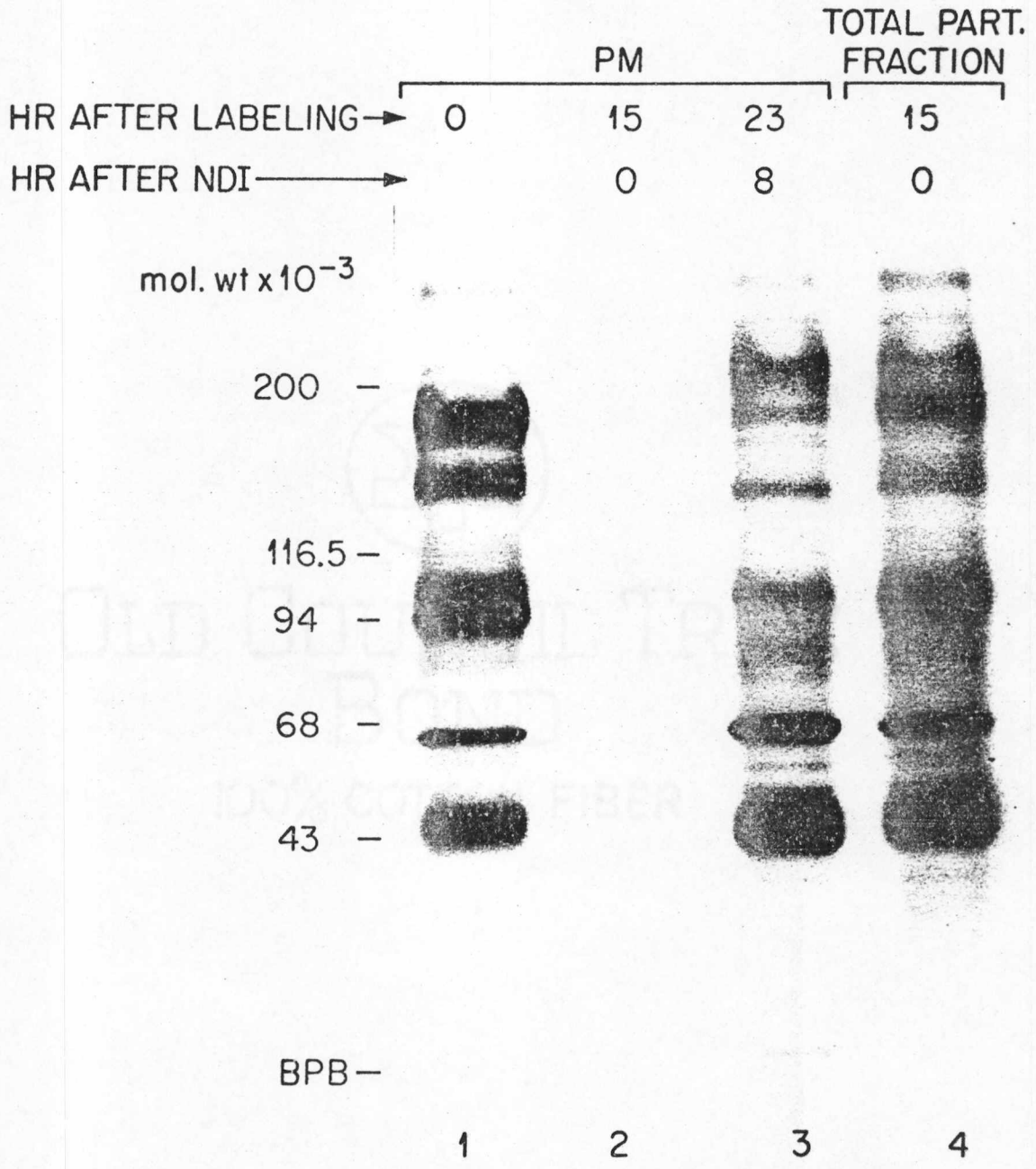

TOTAL PART. FRACTION
PM
HR AFTER LABELING → 0 15 23 15
HR AFTER NDI → 0 8 0
mol. wt x10⁻³
200 –
116.5 –
94 –
68 –
43 –
BPB –
1 2 3 4

Figure 17

labeled as above and plasma membranes isolated immediately after labeling and every 25 h thereafter (Figure 16). At 50-100 h after labeling, the pattern of sialoglycoproteins on the cell surface had changed dramatically, suggesting that preferential treatment of certain sialoglycoproteins had taken place.

Three different types of preferential treatment could have accounted for the changes observed in Figure 16: (1) preferential internalization of certain cell surface components; (2) preferential degradation of cell surface components; and (3) preferential recycling. In an effort to determine which one or more of the above situations accounted for changes that were observed in Figure 16, visualization of recycled material and the intracellular pool was necessary. Recycling was then visualized by labeling cells as above, incubating the cells for 15 hours, during which time substantial internalization of the cell surface occurs, and then treating the cells with neuraminidase. Subsequent reincubation of the neuraminidase-treated cells results in recycling of labeled sialoglycoproteins from an intracellular (neuraminidase-insensitive) pool, back to the cell surface.

As seen is Figure 17, lanes 1 and 4, the material which is found in the total particulate fraction

immediately after this neuraminidase treatment is not equivalent with the material which was present on the cell surface immediately after labeling. Since neuraminidase removes at least 85% of the cell surface-associated radioactivity (Figure 17, lane 2; see also Table I and Figures 5 and 6), this material thus represents the internalized, cell surface sialoglycoproteins present in the intracellular pool and, therefore, the radioactive material available for recycling. Bands 2, 3, 4, and 5 were reduced in their fractional contribution to the total radioactivity located intracellularly, while bands 1, 6 and 7 were increased in intensity. Band 4, it should be noted, was almost completely absent from the intracellular pool. When neuraminidase treated cells were reincubated, and plasma membranes were analyzed after recycling had taken place (lane 3), the pattern observed for recycling material was significantly different from that observed in lane 1 immediately after labeling. On the other hand, the pattern of the material in lane 4 (the intracellular pool) was very similar to the pattern of recycled material. Slight differences were observed, however, between the intracellular material and recycled material including decreased fractional contributions of bands 2 and 5 and the increased contributions of bands 1, 6 and 7 during recycling. This suggested that sorting had taken place

both during the internalization of the cell surface and during functions associated with recycling. In addition, the similarity of the patterns of recycled material in Figure 17 and the material on the cell surface seen in Figure 16 (50 to 100 hours) also supports the fact that neuraminidase, if it is undergoing internalization, does not have a significant effect on the intracellular pool. Experiments outlined below will address the question of whether preferential degradation takes place.

Sorting of Internalized, Surface-Derived Material

Previous work (see Chapter One) had suggested that internalization of the cell surface does not necessarily involve an internalization of all cell surface components. The extent to which sorting takes place in HeLa cells at the level of endocytosis of the cell surface was analyzed using cells preincubated for 8 h in dextran (1mg/ml; see below) which were then labeled and allowed to internalize their cell surface for 60 min at 18°C. The incubation of HeLa cells at 18°C inhibits the fusion of surface-derived vesicles with lysosomal elements (Weigel, 1978; Wolkoff et al., 1984; also see Discussion). The cells were then incubated for 120 min at 18°C at pH 5.0 in the presence or absence of 2 units/ml neuraminidase. After washing the cells twice in cold PBS, the cells were homogenized with

30-40 Dounce strokes in 0.25 M sucrose-10 mM Tris, pH 7.4, and Percoll gradient analysis was performed as described in Chapter Three. Figure 18 illustrates the pattern of radioactivity observed for the control cells and for the neuraminidase-treated cells. The endocytic vesicles, previously shown to sediment at a density of approximately 1.050 to 1.055, were found primarily in fractions 16-18. Contamination by Golgi was minimized by preincubating the cells with dextran 500, previously shown to cause a small increase in the density of the Golgi (Figure 8). This caused the Golgi material to sediment at a density of approximately 1.060-1.065 g/cc, outside the endocytic vesicle peak.

Subcellular fractions from various experiments were analyzed by SDS-PAGE followed by fluorography, and the various lanes were collated in Figure 19A. Figure 19B is the densitometric scan of these various lanes with the Gaussian fits calculated by computer (see Materials and Methods). Fluorographic analysis of fractions 16 to 18 revealed that a number of cell surface bands had been internalized into the endocytic vesicles, but several bands were reduced or absent. In particular, bands 4 through 7 were about the same intensity relative to each other in fraction 17 that they were on the cell surface immediately after labeling (lane 1). A low molecular

Figure 18. Isolation of endocytic vesicles containing radiolabeled, surface-derived sialoglycoproteins. HeLa cells preincubated with dextran, were labeled as described in Materials and Methods and the cells incubated at 18°C for 60 min. Cells were then incubated in fresh medium plus 0.5% fetal bovine serum, pH 5.0, in the presence of neuraminidase, as described in Materials and Methods. The cells were then washed, homogenized, and a post-nuclear supernatant isolated. This supernatant was layered over 30% Percoll-0.25 M sucrose, and centrifuged at 17,000 RPM for 30 min in a 50 Ti rotor. This gradient and a control gradient treated in the same way but in the absence of neuraminidase, were fractionated, and various fractions assayed for TCA-precipitable radioactivity. Since the neuraminidase removes only about 85% of the cell surface radioactivity (see Chapter II) the contamination of the endocytic vesicle radioactivity with cell surface-derived radioactivity was calculated, and is represented by the cross-hatched area. More than 85% of the radioactivity contained in fractions 16 through 18 could be attributed to radioactivity located intracellularly.

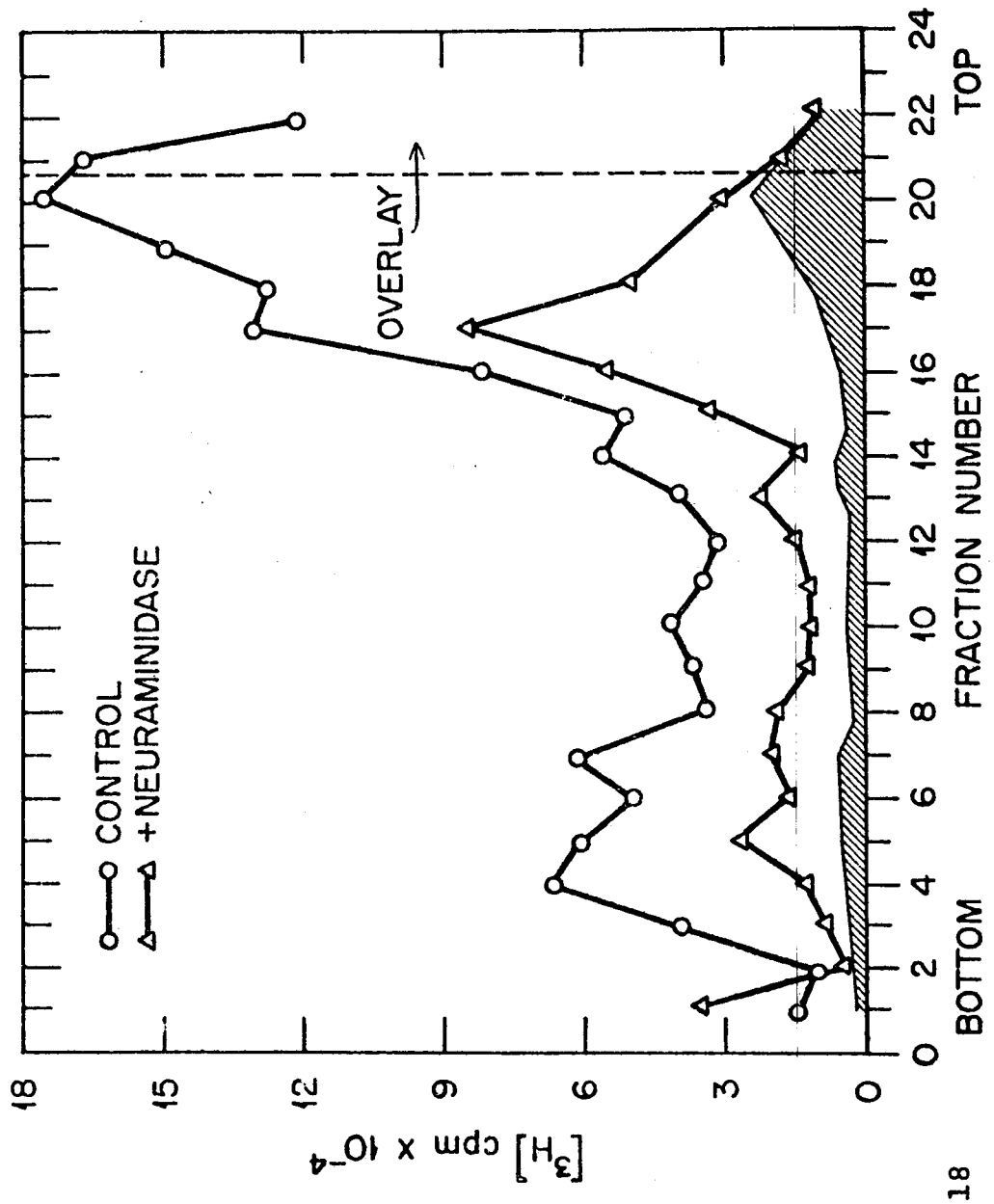

CONTROL
+NEURAMINIDASE
OVERLAY
[3H] cpm x 10-4
FRACTION NUMBER
BOTTOM
TOP
0
3
6
9
12
15
18
0
2
4
6
8
10
12
14
16
18
20
22
24

Figure 18

weight band also appearred towards the bottom of the lane (denoted by an asterisk), most likely a breakdown product caused by extracellular or cell surface proteases. The pattern was somewhat different in fractions 16 and 18. This difference could be attributed to the isolation of several different types of vesicles, or it could have been vesicles of various densities related temporally (see Discussion). Whatever the relationship might be, it is apparent that sorting had taken place between the cell surface and the formation of the intracellular vesicles shown in Figure 19. Further speculation regarding sorting at the level of the endosome or endocytic vesicle is not possible from the available data.

As was described in Chapter III , the internalized surface sialoglycoconjugates associate with the lysosomes, and can then be transferred to the Golgi complex. It was, therefore, of interest to characterize further these fractions enriched for lysosomes and Golgi at various times after cell surface labeling. In Figure 19 (A and B), lanes 5 through 7, the fluorographic pattern of Golgi and lysosomal fractions isolated as described in Chapter III were compared to the fluorographic patterns observed for the endocytic vesicles and plasma membranes isolated immediately after labeling or isolated after recycling had taken place (see also Figures 6 and 16). The lysosomal

Figure 19. SDS-PAGE/fluorographic analysis of cell surface-derived sialoglycoproteins associated with various subcellular fractions. Subcellular fractionation was performed as described in Chapter III for the isolation of lysosomes, plasma membranes, and Golgi complex, and the fractions analyzed by SDS-PAGE followed by fluorography. In addition, endocytic vesicles, isolated as described in the legend to Figure 18 were also analyzed, along with the intial labeling from Figure 16 included again as a reference in lane 1. The gradients were 4-10% acrylamide. A. Fluorogram photographed from Kodak XAR film. The arrowheads represent the approximate positions of the seven major bands and the asterisk marks presumably a breakdown product (see Discussion). B. Densitometric scans of the film, with curves fit by computer (see Materials and Methods). Due to size restriction of the scanner, the very bottom of the film (below approximately 30,000 mol. wt.) was not scanned.

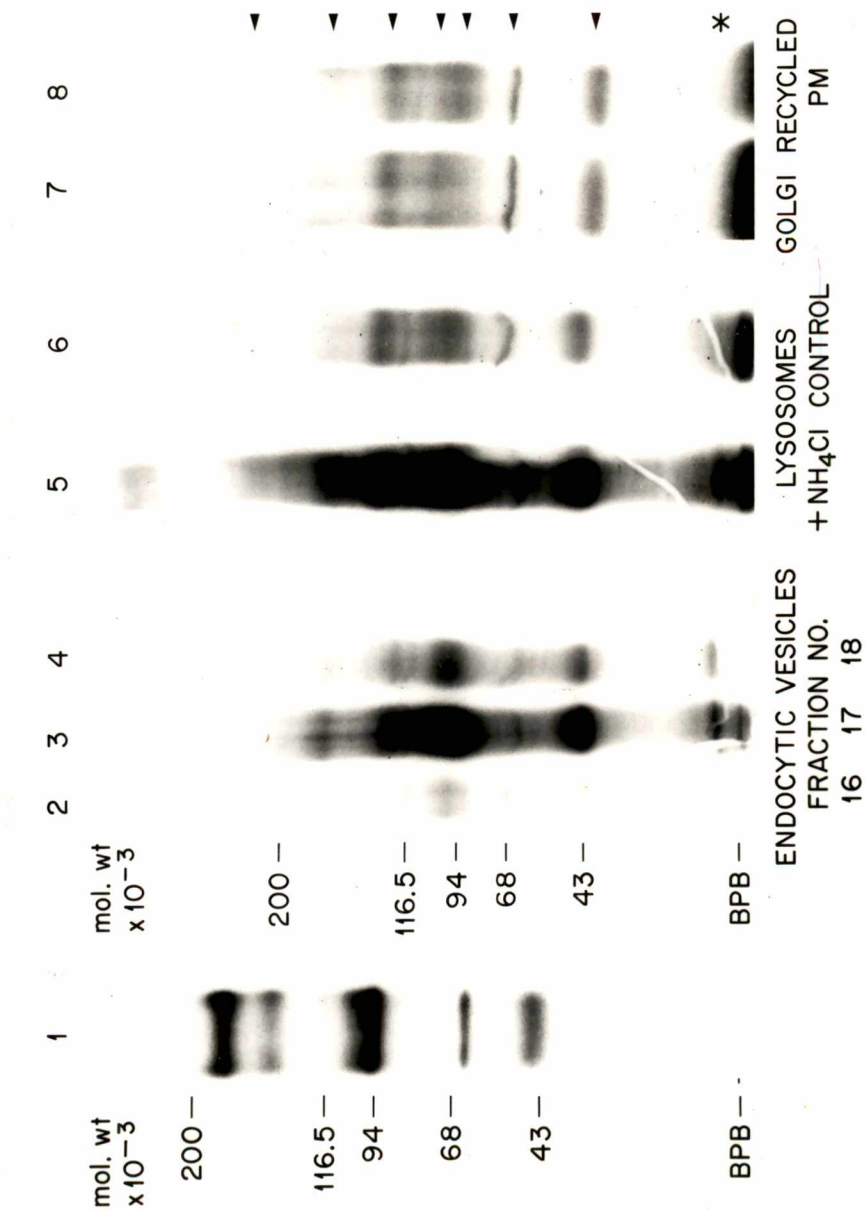

1
2 3 4 5 6 7 8
mol. wt
x10⁻³
200 —
116.5 —
94 —
68 —
43 —
BPB —
ENDOCYTIC VESICLES
FRACTION NO.
16 17 18
LYSOSOMES
+NH4Cl CONTROL
GOLGI RECYCLED
PM

Figure 19

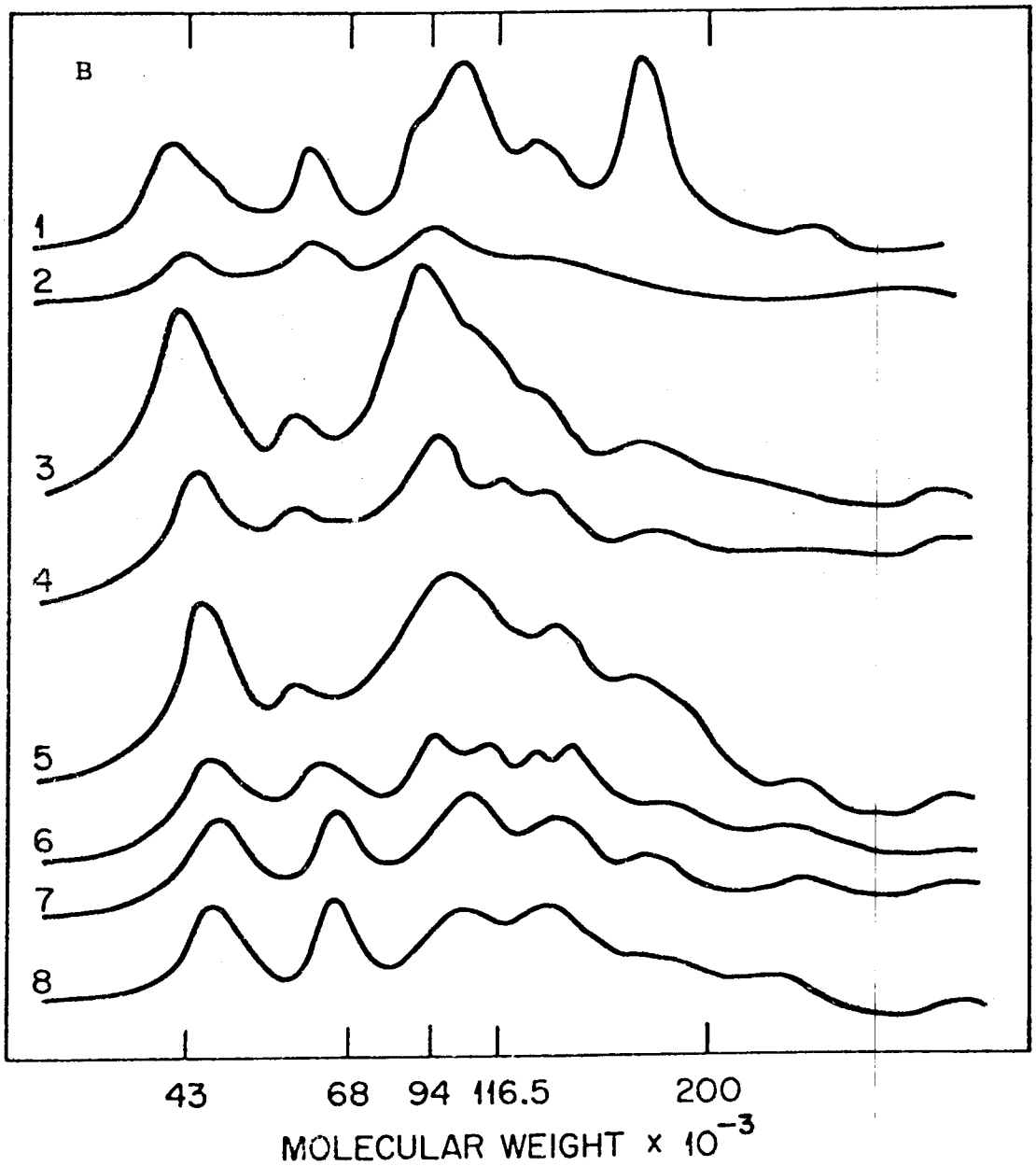


Figure 19, continued

fractions (lanes 4 and 5), particularly the lysosomes from the NH_4Cl treated cells, more closely resemble plasma membranes isolated immediately after labeling than those isolated after recycling had taken place. Several major differences can be observed between the lysosomes isolated from untreated (control) cells and those isolated from NH_4Cl treated cells. In the presence of NH_4Cl, the fluorographic pattern of sialoglycoproteins was virtually identical to that observed for the endocytic vesicle peak in fraction 17 (lane 3). In control lysosomes the pattern was significantly different, with both band 2 and the low molecular weight band, denoted by the asterisk, notably absent, the latter presumably degraded (see Discussion). In lane 7, the fluorographic pattern of the material isolated from Golgi fractions is shown, which looks virtually identical to the pattern for material undergoing recycling shown in lane 8. The pattern of radioactivity associated with the various fractions is summarized in Table IV. The pattern of radioactivity in the endocytic vesicles in fraction 17 (lane 3) is quite similar to what was seen associated with the lysosomal compartment. Certain other sialoglycopeptides and/or sialoglycoproteins appear to be lost from the lysosomes in control versus treated cells (lanes 5 and 6) suggesting that either degradation or an inhibition in lysosome to Golgi transfer as seen in Figure

TABLE IV

Integration of Densitometric Scans

The densitometric scans of the fluorogram in Figure 19A were analyzed and fit to Gaussian curves by the GELSCAN program provided by LKB instruments. The curves were fit to less than 5% error, and the peak areas of bands 2 through 6 are summarized below as the percentage of the total radioactivity present relative to the total in bands 2 through 6. Band 1 was not included in this tabulation due to its extremely low level of radioactivity in these experiments.

Lane Number	Band Number 7	6	(4 + 5)*	3	2
1	24.0	7.6	37.8	26.7	4.1
2	16.9	10.5	47.3	24.9	0.6
3	33.0	7.9	45.1	9.2	4.7
4	27.0	5.6	43.0	16.4	2.2
5	23.3	7.9	47.5	10.4	11.0
6	29.8	7.1	31.6	26.6	5.0
7	29.0	9.6	34.6	22.0	4.8
8	29.5	8.8	30.6	20.1	11.0

* Bands 4 and 5 could not be sufficiently differentiated to determine accurately their individual contributions to the total radioactivity. Their densities have, therefore, been summed.

14 may be responsible. Direct return to the cell surface from the lysosomal compartment is unlikely to be a very important component of sialoglycoprotein recycling in HeLa cells due to the time course of loading of surface-derived material into the various subcellular fractions and the comparison of this information with the time course for recycling presented in Chapter II. The majority of the material which undergoes the long-term recycling discussed in Chapters II and III is found in bands 3, 5, 6 and 7. These bands comprise the majority of the Golgi-associated, surface-derived material as well as the majority of the material which recycles back to the cell surface. Taken together with the data that was presented in Chapter III, the above data reinforce the argument that these bands are internalized via endocytic vesicles, then pass through the lysosomal compartment and are then transferred to the Golgi complex prior to recycling. The similarity of the SDS-PAGE fluorographic patterns between the Golgi enriched fractions and the recycled material also suggests that this sorting may be complete once internalized surface components reach the Golgi complex.

The movement of the sialoglycoproteins described in Chapters III and IV are summarized below in Figure 20. Even though recycling from endocytic vesicles was not investigated here, it is a well-documented phenomenon

Figure 20. Pathways of intracellular sorting and the transport of cell surface sialoglycoproteins in HeLa cells. The various pathways outlined in Chapters III and IV for the internalization and intracellular movement of internalized sialoglycoconjugates are summarized. The abbreviations are: EV, endocytic vesicle; CV, coated vesicle; E, endosome; 1^{o}L, primary lysosome; 2^{o}L, secondary lysosome; GC, Golgi complex; ER, endoplasmic reticulum; and N, the nucleus. The internalization and the recycling of surface-derived sialoglycoconjugates involves: (1) internalization of the cell surface via endocytic or coated vesicles; (2) these can fuse to form a larger structure, the endosome; (3) material can recycle back to the cell surface prior to the fusion of the endosomes with primary lysosomes (4), forming a secondary lysosome; (5) sorting can take place in the secondary lysosome in the form of degradation, and also via recycling in some cases (6), though no evidence to support such a pathway of recycling for surface-derived sialoglycoproteins was found here; (7) the transfer of material can take place from the lysosomes to the Golgi complex, and then to, perhaps, a specialized region of the Golgi known as the GERL; and (8) the recycling of internalized, surface-derived sialoglycoproteins takes place from the Golgi to the cell surface.

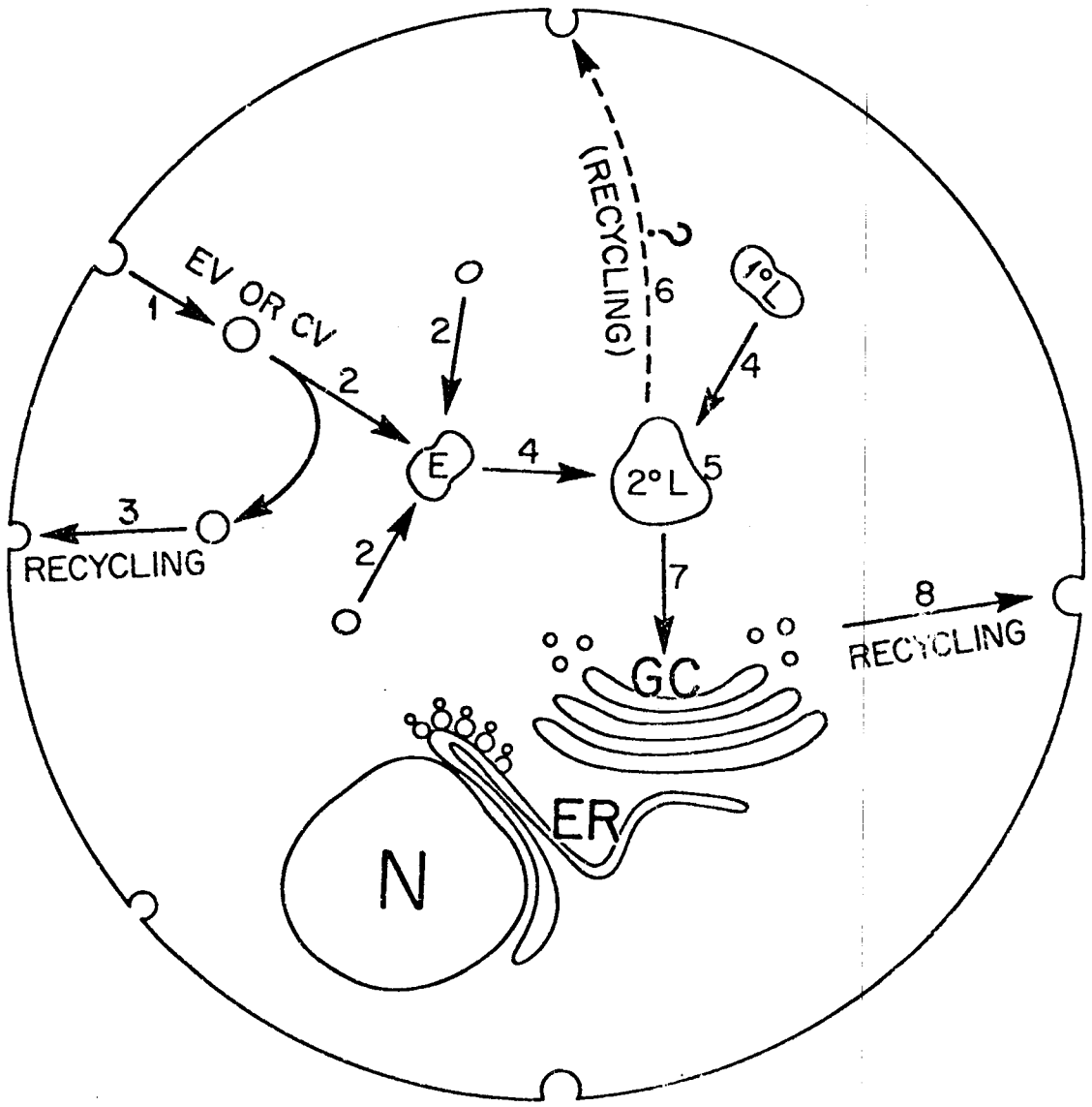

EV OR CV
1
2
RECYCLING
3
E
2
2
4
2°L
5
1°L
4
(RECYCLING)
?
6
7
GC
8
RECYCLING
ER
N

Figure 20

(Pastan and Willingham, 1981; Bridges et al., 1982; Baenziger and Fiete, 1982; Wolkoff et al., 1984), and has been included in this figure.

Discussion

The phenomenon of the recycling of internalized cell surface components has been the subject of much recent work. Several methods have been employed to detect such movement, including antibody detection (Schneider et al., 1979b; Louvard, 1980; Widnell, 1982), protease treatment (Stahl et al., 1980), and kinetic analysis (Ciechanover et al., 1983; Schwartz et al., 1984). These methods, while useful for demonstrating the existence of recycling, have serious drawbacks when used for quantitative analysis or investigation into the intracellular pathway(s) involved in recycling (see Chapter I for details). In an effort to quantitate recycling and to establish routes of the internalization and the externalization of cell surface components, I have modified the labeling technique of Van Lenten and Ashwell (1971) and have used it to label cell surface sialoglycoconjugates. Further analysis by subcellular fractionation and the fluorographic analysis of the surface derived material contained in these fractions and in other fractions that were isolated above in Chapter II

at various times after labeling has allowed for the establishment of sorting sites and routes of movement of internalized, cell surface sialoglycoproteins.

Internalization and Intracellular Sorting of Cell Surface Sialoglycoproteins

The time course of recycling of cell surface-derived sialoglycoconjugates, as outlined in Chapter II, has a halftime of approximately 3-5 hours. In an effort to further determine the extent to which cell surface sialoglycoproteins undergo recycling, the fluorographic pattern of surface sialoglycoproteins immediately after labeling was compared to the pattern seen intracellularly after internalization and to the pattern of material recycling. Changes in the fractional contribution of each radiolabeled band to the overall pattern were seen when comparing the internalized material with the original labeled surface. Other minor changes were also seen when comparison is made between the intracellular pool and the recycled material. These changes could have occurred at several levels of cellular organization: (1) preferential internalization or microdomains, such as coated pits on the cell surface, could result in sorting taking place during the process of internalization; (2) sorting at the level of endocytic vesicles and endosomes may result in preferential movement of material to other intracellular

organelles (e.g., to the lysosomes or back to the cell surface); (3) The degradation of certain surface-derived components in the lysosomes, while others are spared; and (4) preferential recycling of certain components from intracellular locations.

The labeling technique described here has allowed for the elucidation of the relative contributions of each of these possibilities to the overall pattern of recycling observed above. The use of neuraminidase in conjunction with low temperature endocytosis (Figure 18) allowed for the visualization of internalized surface components in endocytic vesicles. Three fractions of endocytic vesicles were analyzed, each with different flurographic patterns of surface-derived material. It is obvious, from the patterns shown in Figure 19, that differential internalization (i.e., sorting which occurs at the level of internalization) has taken place. I have referred to this material as endocytic vesicles, though there is no clear cut evidence to suggest that they are not endosomes (or even receptosomes). Indirect evidence in favor of this material being endocytic vesicles (versus endosomes) may be inferred from the fact that at 18^{o}C, the fusion of internalized vesicles with the lysosomes as well as with the plasma membrane is inhibited. Other studies have previously demonstrated that above 21 to 24^{o}C, there is a

break seen in the curve of the diffusion of fluorescent membrane probes (Jacobson et al., 1981), a sharp increase in acyl chain mobility (Mendelsohn et al., 1982), as well as an increase in vesicle fusion (Enoch et al., 1977). These results were observed in synthetic bilayer vesicle preparations (Enoch et al., 1977), vesicles prepared from isolated membrane lipids (Jacobson et al., 1977) as well in cells that were allowed to take up a hydrophobic fluorescent probe (Jacobson et al., 1977). These results suggested that the inhibition of internalized cell surface vesicle fusion with the lysosomes and plasma membrane at 18°C may be due to the membrane bilayer being in a semi-solid rather than in a liquid-crystalline state (i.e., 18°C being below the phase transition temperature). This would inhibit virtually all vesicle fusion, and would result in the accumulation of endocytic vesicles, rather than endosomes which are the product of fusion of many endocytic vesicles. On the other hand, the pinching off of a vesicle at the cell surface might be aided by the presence of clathrin and other intracellularly located clathrin associated proteins (Pearse, 1982; Daiss and Roth, 1983) which may provide enough energy to overcome the above temperature effect (Fagan and Dewey, 1984).

The subcellular fractionation studies outlined in Chapter III demonstrated the movement of internalized,

surface-derived material into the lysosomes reaches its peak approximately 1 hour after labeling. Lysosomes were isolated from cells one hour after labeling. One group of cells had been treated with NH_4Cl, which was previously shown to raise the intralysosomal pH and disrupt the lysosomally-dependent degradation (DeDuve et al., 1974; Ohkuma and Poole, 1978; Christopher and Morgan, 1981; see also Figure 12). Comparison of these lysosomes to the pattern of surface derived material seen in the endocytic vesicles, in particular fraction 17, shows that the lysosomes isolated from the cells treated with NH_4Cl contained virtually the same pattern of sialoglycoproteins. The absence of NH_4Cl resulted in a much different pattern of sialoglycoproteins, with the notable absence of several bands, including a low molecular weight breakdown product. It is unlikely that these missing bands were transferred out of untreated lysosomes, because they do not reappear in the Golgi or on the cell surface. Undoubtedly, the movement of material through the lysosomal compartment results in sorting in the sense of degradation of certain, degradable surface derived components, and the transfer of other cell surface derived components to other organelles. Direct recycling from the lysosomal compartment itself is unlikely to account for significant recycling of

sialoglycoproteins due to the time course of recycling reported in Chapters II and III.

The time of uptake of surface derived material into the Golgi complex was seen to occur between 1 and 3 h after surface labeling, and was inhibited by NH_4Cl or chloroquine (Figure 14). The overaccumulation of material within lysosomes during the above treatment suggested that material was transferred from the lysosomes to the Golgi complex. The pattern of radiolabeled sialoglycoproteins in the Golgi was qualitatively very similar to that seen in the lysosomes not treated with NH_4Cl. In addition, the pattern of recycled material was virtually identical with the pattern of radiolabeled surface material associated with the Golgi. It thus seems likely that the sorting of internalized, cell surface material takes place at both the level of the cell surface and during the association of internalized material with the lysosomes. The similarity of the pattern of the Golgi and recycled, surface radiolabeled sialoglycoproteins suggests that sorting may be complete once lysosomal sorting (i.e., degradation) is complete.

The above data cannot conclusively rule out other pathways being involved in the processes of cell surface internalization, recycling, and sorting, but in the case of sialoglycoproteins, the pathways outlined above seem to

involve at least the majority of the cell surface-derived sialoglycoproteins.

CHAPTER V

IMPLICATIONS OF CELL SURFACE SORTING AND RECYCLING

Cellular regulation of the concentration of cell surface components is known to occur at the levels of transcription and translation. In light of the data presented here and together with that published by other laboratories (Schneider et al., 1979; Louvard, 1980; Stahl et al., 1980; Widnell et al., 1982; Bleil and Bretscher, 1982; Schwartz et al., 1984) it is likely that such regulation also occurs at the levels of sorting and recycling. Cells seem to have the capacity to remove certain cell surface components selectively, eliminating the need for large scale degradation and biosynthesis to alter the composition of the cell surface.

Much of the published information on recycling has dealt with individual surface proteins in certain cell types, with the information, obviously, being applicable only in the broadest sense to other cell types and other surface components. The utilization of a labeling method such as that described here allows for a more general study of the cell surface as a whole, and thus results applicable to many cell types can be derived from such

data. It seems evident from the data presented here, that all labeled cell surface sialoglycoproteins are first internalized into a definable endocytic compartment, and that this compartment transfers at least some of its material to the lysosomes. Until recently, the lysosomal compartment had been thought of as a degradative dead-end, but it now seems clear that degradation may play a key role in the maintenance of the integrity of cell surface components. Several bands of radioactive material which were internalized into lysosomes and visulaized under conditions where the degradative process was impaired, were not present in untreated cells. Though this might have been due to the transfer of this material to other cellular compartments, these bands did not reappear in any other compartments analyzed, suggesting they were degraded upon association with the lysosomal compartment. Though further transfer of this material takes place from the lysosomes to the Golgi complex, there does not seem to be any further sorting of material at this step or during recycling.

The importance of degradation in the maintenance of the cell surface and the sorting of various cell surface components cannot be underestimated. Turnover of specific proteins may be necessary to ensure the proper functioning of the cell under certain conditions, and could be

achieved via preferential internalization or degradation. In the case of certain cell surface receptors, such responses can be quite rapid. Internalization of 95% of the cell surface epidermal growth factor receptors is seen in minutes upon the addition of nanomolar concentrations of EGF to the extracellular medium (Wiley and Cunningham, 1983). Down regulation of these receptors would result in an almost immdeiate cellular insensitivity to further EGF addition, and an insenstivity to other components which seem to act synergistically with EGF at the level of the receptor (Cochet et al., 1984; Iwashita and Fox, 1984; Oleshaw et al., 1984). Specific sorting and recycling may also allow for the up-regulation of certain cell surface components. Unlike alterations in gene expression, which for plasma membrane proteins may represent a lag of many hours prior to up-regulation (Pollack et al., 1981b), the presence of an intracellular pool of surface-like material could enable a cell to increase its level of a certain protein by specific externalization of various components. This has been shown to be the case for the mobilization of insulin receptors in fat pad cells (Suzuki and Kono, 1980), and may also have wide spread implications in other systems where physiological up-regulation is important to maintain cellular homeostasis (for example see Pollack et al., 1981b).

The importance of maintaining the proper balance of all cell surface components cannot be comprehended by the information garnered from a single receptor system or a single cell surface component. The treatment of the entire cell surface must be examined in an effort to fully understand the ramifications of internalization, sorting and recycling. The widespread use of any cell surface labeling techniques such as the one described here can only help but improve our understanding of cellular control mechanisms.

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