

Density of Newly Synthesized Plasma Membrane Proteins in Intracellular Membranes II. Biochemical Studies

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ABSTRACT Using two independent methods, incorporation of radioactive amino-acid and quantitative immunoblotting, we have determined that the rate of synthesis of each of the Semliki Forest virus (SFV) proteins in infected baby hamster kidney (BHK) cells is 1.2×10^5 copies/cell/min. Given the absolute surface areas of the endoplasmic reticulum and Golgi complex presented in the companion paper (Griffiths, G., G. Warren, P. Quinn, O. Mathieu Costello, and A. Hoppeler, 1984, *J. Cell Biol.* 98:2133–2141), and the approximate time spent in these organelles during their passage to the plasma membrane (Green J., G. Griffiths, D. Louvard, P. Quinn, and G. Warren 1981, *J. Mol. Biol.* 152:663–698), the mean density of each viral protein in these organelles can be calculated to be 90 and 750 molecules/ μm^2 membrane, respectively. In contrast, we have determined that the density of total endogenous integral membrane proteins in these organelles is $\sim 30,000$ molecules/ μm^2 so that the spike proteins constitute only 0.28 and 2.3% of total membrane protein in the endoplasmic reticulum and Golgi, respectively. Quantitative immunoblotting was used to give direct estimates of the concentrations of one of the viral membrane protein precursors (E1) in subcellular fractions; these agreed closely with the calculated values. The data are discussed with respect to the sorting of transported proteins from those endogenous to the intracellular membranes.

We have used the two membrane-spanning (spike) glycoproteins (E1 and p62) of Semliki Forest virus (SFV)¹ as probes to follow plasma membrane (PM) proteins from their site of synthesis in the rough endoplasmic reticulum (ER) to the cell surface. During transport they must be separated from the endogenous proteins of the membranes they pass through, and concentrated with respect to the membrane. There are at present no quantitative data available even to define the extent of these processes.

In the accompanying paper, we have introduced the approach for estimating the densities (number of molecules per unit surface area) of the spike proteins of SFV in the ER and Golgi membranes. The absolute surface areas of ER, Golgi, and plasma membrane of an "average" baby hamster kidney (BHK) cell were estimated by stereological procedures. One aim of this paper is to determine the total number of molecules of SFV spike proteins in the membranes of the ER and the Golgi complex of an average infected BHK cell so that their densities can be calculated. The density of spike proteins

in mature virions is already known, and direct comparison of the densities at various stages of intracellular transport can therefore be made. For direct measurement of the densities of viral protein in subcellular fractions, it is also necessary to know the total concentration of integral membrane proteins in the membranes of ER, Golgi, and plasma membrane. This is the second aim of this paper.

MATERIALS AND METHODS

Materials, cell culture, viral infection, and labeling of cells were as previously described (14).

Cell Counting: In each experiment, six extra dishes of cells were plated out, grown, and infected in parallel with the experimental cultures. Cell number was determined using a haemocytometer after releasing the cells by incubation with trypsin/EDTA. In some cases, cell number was determined directly by taking random photographs of the cells in situ using a $\times 25$ Zeiss water immersion lens. Cells were counted on the photographically enlarged pictures at a final magnification of $\times 100$. The results from the two methods agreed within 10%.

Quantitative Immunoblotting: Samples of cells or subcellular fractions were prepared for electrophoresis as described (14). Media samples were concentrated by precipitation with trichloroacetic acid (10% wt/vol final con-

¹ Abbreviations used in this paper: BHK, baby hamster kidney cells; ER, endoplasmic reticulum; SFV, Semliki Forest virus.

centration). After 1 h at 4°C, the precipitates were collected by microcentrifugation. They were washed twice with ethanol/ether (1:2 vol/vol), then with ether, and allowed to air dry before being dissolved in electrophoresis sample buffer.

After electrophoresis on polyacrylamide gels, proteins were transferred to nitrocellulose filters according to the method of Towbin et al. (32), and modified as described by Burnette (6) and Griffiths et al. (15). Protein was visualized by incubation with antiserum to E1, followed by iodinated protein-A and autoradiography. Quantitation was achieved by excizing the radioactive bands with a scalpel and counting them directly in an NE1600 gamma counter. As described by Howe and Hershey (18), counts were linear with added antigen in the range 5–50 ng. In all cases, three standards were run on the gels to allow correction for variations caused by individual incubation conditions.

Subcellular Fractionation: This was performed as described by Renkonen et al. (24) or by Green et al. (14). The two procedures gave similar results for ER and plasma membrane. No Golgi-derived material was obtained using the former method. Washing with sodium carbonate was as described by Howell and Palade (19). The method essentially removes all luminal content of membrane vesicles and most of the peripheral membrane proteins (20). In this paper, we assume that the remaining proteins are integral, either spanning the bilayer or having a portion deeply embedded in it.

Estimation of Phospholipid and Cholesterol: Lipid extraction was performed as described by Bligh and Dyer (4). Total phosphorus was determined by the method of Rouser et al. (28) and cholesterol by the cholesterol ester-cholesterol oxidase reaction (Boehringer, Mannheim).

Estimation of Protein and Mean Molecular Weight: Total protein was measured using the Biorad reagent (Biorad Laboratories, München, Federal Republic of Germany) with immunoglobulin standards, or by the micro-Kjeldhal technique, modified as described by Jaenike (21) using ammonium sulphate standards. Results from the two methods agreed within 5%.

Protein molecular weights were determined on 10% SDS-polyacrylamide gels. These were stained with Coomassie Brilliant Blue (SERVA, Heidelberg, Federal Republic of Germany) and photographed with transmitted light using Agfa ortho film. 35-mm negatives were scanned on an Optronix P-1000 densitometer using a 50- μ m band width and the data analyzed by computer (31). Estimation of mean molecular weight of proteins in the sample was from computer calculation of the number of molecules at each data point and assigned a molecular weight by reference to protein standards (Biorad Laboratories) run in a parallel track.

RESULTS

To obtain figures for the total number of viral proteins in the ER and Golgi we have taken advantage of the fact that the approximate time spent in these organelles by the viral membrane proteins can be calculated from published data (14). It is therefore possible to calculate the average number of molecules present from the rate of synthesis of the viral proteins.

Rate of Synthesis of Viral Proteins

Synthetic rates can be estimated in a number of ways and we have used two completely independent methods to preclude significant systematic errors.

MEASUREMENT BY INCORPORATION OF RADIOACTIVE AMINO ACIDS: We have measured the incorporation of [³⁵S]methionine into protein by SFV-infected BHK cells, using a constant amount of [³⁵S]methionine and a range of concentrations of unlabeled methionine. Incorporation was allowed to proceed for only 15 min to exclude the possibility of loss of labeled protein from the cells by viral budding. The results are shown in Fig. 1A. Linear regression analysis of the data points using more than 5 μ g carrier methionine gave a best fit line of zero slope, indicating that the calculated synthetic rate was independent of the specific activity of the added methionine over this range. The intercept value is 2.59×10^6 molecules of methionine incorporated/cell/min.

As the measured rate of synthesis was unaffected by the

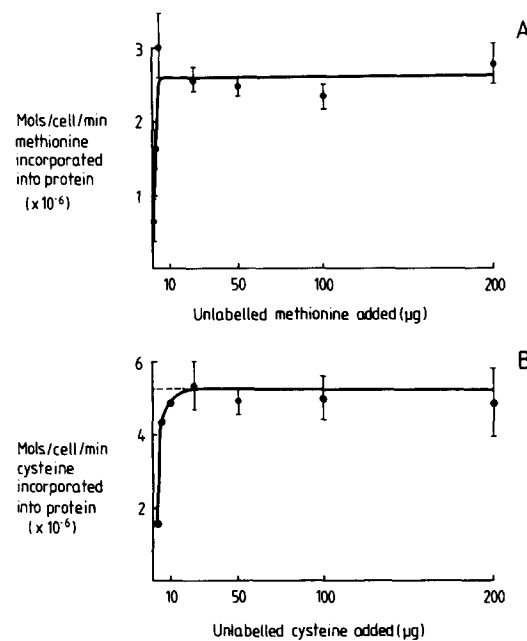


FIGURE 1 Incorporation of amino acids into protein by BHK cells infected with SFV. Cells, 6 h post infection, were labeled for 15 min with (A) 50 μ Ci [³⁵S]methionine and various amounts of unlabeled methionine, or (B) 50 μ Ci [³⁵S]cysteine and various amounts of unlabeled cysteine. After washing the cells, radioactivity incorporated into TCA-precipitable material was determined and used with the specific activity to calculate the amount of amino acid incorporated. Data points are means of duplicate determinations from each of three dishes of cells.

specific activity of the labeled methionine used (provided that there was more than 5 μ g unlabeled methionine present), it is clear that the estimate of synthetic rate is not distorted by large intracellular pools of unlabeled methionine, nor by the equilibration of external and internal pools being slow in comparison with the period of labeling. BHK cells infected with SFV are essentially making only viral proteins. These are made in approximately equimolar amounts from a single mRNA, of which the complete coding sequence is known (13). In this case, then, the extrapolation from moles of methionine incorporated to number of proteins synthesized can be made, and gives a figure of 0.96×10^5 translations of the viral 26S mRNA, per cell, per minute.

The experiments were repeated using [³⁵S]cysteine as the label and very similar results were obtained (Fig. 1B). As with the methionine incorporation, the best line through data points using more than 5 μ g unlabeled amino-acid had zero slope. The rate of synthesis, equivalent to 1.09×10^5 copies of each viral protein/cell/min agrees well with the previous estimate.

The rates of synthesis at various times after infection are shown in Fig. 2. From 4 h postinfection, the rate stayed constant for 4 h, and was still 80% of maximum at 14 h. Even at 20 h, when the cells had begun to die, the rate had only dropped by 50%.

MEASUREMENT BY IMMUNOBLOTTING: To quantitate the rate of production of E1, samples were taken from cells and their media at different times after infection. These, together with standard amounts of E1, were run on polyacrylamide

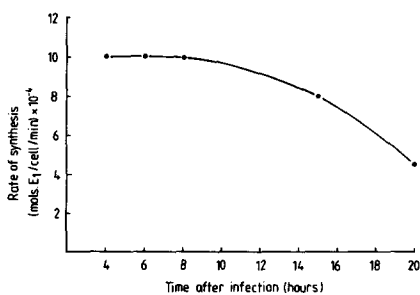
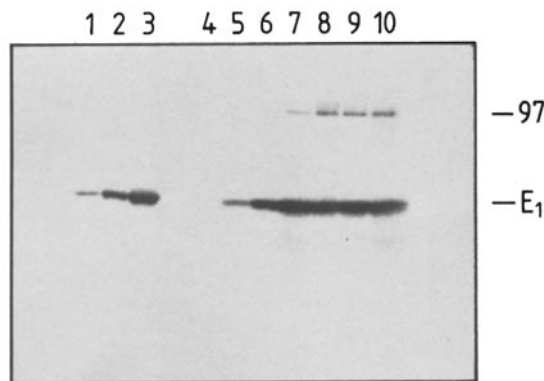


FIGURE 2 Rate of viral protein synthesis as a function of time after infection. At each time point, the synthetic rate was estimated as described in Fig. 1 but using only 20, 50, and 100 μg of unlabeled methionine.



Time (h) post infection: 0 4 6 8 10 14 20

FIGURE 3 Immunoblot of a polyacrylamide gel, visualized with antiserum to E1 and iodinated protein A. Samples were standards (lanes 1-3) calculated to contain 10, 25, and 50 ng E1, respectively) and extracts of BHK cells at different times after infection with SFV (lanes 4-10). The 97-Kdalton protein (97) is an aberrant transcript containing E1, always seen in infected cells.

gels. Proteins were transferred to nitrocellulose filters, which were incubated with anti-E1 followed by iodinated protein A. Radioactive bands were localized by autoradiography, excized, and counted. A typical autoradiogram is shown in Fig. 3. To minimize cross contamination, only alternate lanes on the gel were used. It was found, in agreement with Howe and Hershey (18), that bound protein A increased linearly with amount of antigen loaded between 5 and 50 ng. At higher levels, amounts could be estimated by reference to a standard curve, but with much increased error. The amount of sample assayed was therefore adjusted to give amounts of E1 in the linear range. The results obtained from such assays are shown in Fig. 4. There is significant variation between the separate samples measured at each time point and the standard errors are correspondingly large.

The isotope incorporation data obtained between 4 and 14 h after infection gave a constant rate of synthesis. Linear regression analysis of the immunoblotting data over this time period gives a best fit line of slope 1.42, corresponding to synthesis of 1.42 μg E1/dish/h. This is equivalent to 1.39×10^5 molecules E1/cell/min synthesized during this period.

Given the considerable variation in estimates for each data point, the two independent methods of measuring the rate of synthesis of the viral proteins agree remarkably well. The mean value, 1.2×10^5 copies of E1/cell/min is used as the best estimate of synthetic rate in the calculations that follow.

Density of Endogenous Protein in Intracellular Membranes

To provide a reference point for subsequent estimation of E1 abundance in the intracellular membranes of infected cells, it was necessary to establish the density of host cell membrane proteins. If the average molecular weight of the proteins is known, the number of protein molecules can be determined. The area occupied by the associated lipid can be calculated because a molecule of phospholipid is known to occupy 63 \AA^2 , and a molecule of cholesterol 35 \AA^2 (8, 11, 22, 33).

Subcellular fractions enriched in ER, Golgi, and plasma membranes were carbonate washed (19) to remove luminal and peripheral proteins. The amounts of lipid and protein found are given in Table I. The mean molecular weight of the proteins in each fraction was calculated from scans of polyacrylamide gels. Each sample was run at several concentrations to allow estimation of each fraction at a loading that was within the range over which protein concentration and optical density on the film negative were linear. Representative scans are shown in Fig. 5. The mean molecular weights

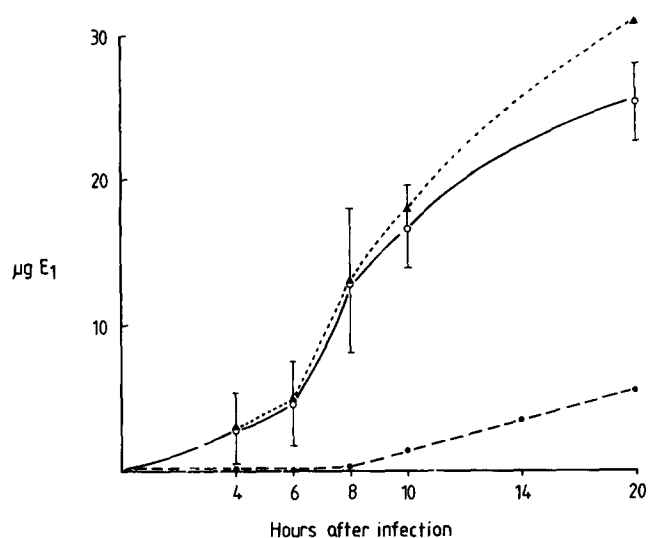


FIGURE 4 Quantitation of E1 at various times after infection. At each time point, the supernatant solution from each of three dishes of cells was collected and precipitated with TCA, and the cells were dissolved in 2% SDS, 100 mM Tris pH 8.8. Each sample was then prepared for SDS PAGE. The amount of E1 in each sample was determined by quantitative immunoblotting. O, E1 in cells; ●, E1 in supernatant solutions, ▲, total E1.

TABLE I
Concentration of Endogenous Proteins in Carbonate Washed Subcellular Fractions of BHK Cells

	ER	Golgi	Plasma membrane
Protein	100	100	100
Mean molecular weight*	46	49	51
Phospholipid	136	130	144
Cholesterol	9.8	19.9	33.6
Area occupied by lipid	3.1	3.2	3.9
Protein density	4.2	3.8	3.0

Units of measure: protein, phospholipid, and cholesterol, micrograms; the area occupied by lipid, $\mu\text{m}^2 \times 10^{-10}$ (calculated); and protein density (molecules/micron squared lipid area) $\times 10^{-4}$.

* $\times 10^3$.

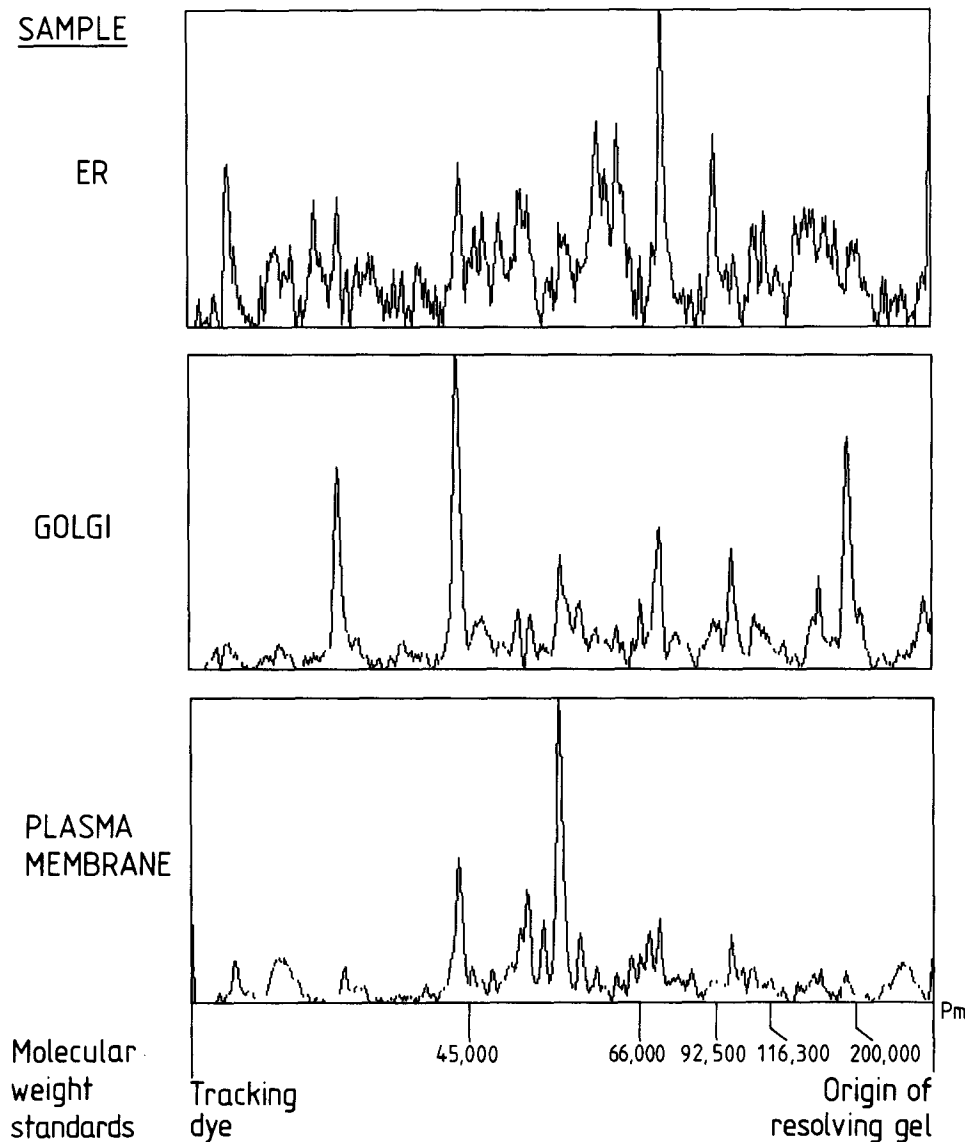


FIGURE 5 Computer printout of scans of tracks from a polyacrylamide gel of carbonated washed subcellular fractions of BHK cells. These outputs have only half the resolution of the data analysis. The mean molecular weight for proteins in each track was determined by computer integration of the amount of protein and molecular weight (determined from the standards on a parallel track) at each data point.

and the consequent estimates of the density of membrane proteins are given in Table I.

Density of E1 in Intracellular Membranes of Infected Cells

Values for the density of E1 in the rough ER and Golgi membranes of infected cells can be obtained both by calculation from the data obtained from whole cells, and by direct measurement of the protein in isolated subcellular fractions.

CALCULATED DENSITIES: The data given above on the rate of synthesis of E1 can be used, with the knowledge of the time taken for the molecules to pass through each organelle (14) and the morphometric data on the surface area of the organelles in the accompanying paper (16) to calculate the density of E1 in the rough ER and Golgi membranes. This data is summarized in Table II. Clearly the viral protein is very dilute at its site of synthesis, and is concentrated by several orders of magnitude during transport.

DIRECT MEASUREMENT OF E1 IN SUBCELLULAR FRACTIONS: Subcellular fractions were prepared from infected cells, and samples subjected to quantitative immunoblotting as described above. The results are presented in Table III, and are in excellent agreement with the calculated values of E1

TABLE II
Calculation of E1 Concentration in Intracellular Membranes

Membrane	Surface area μm^2	Protein dwell time <i>min</i>	No. of E1 mols	Concentration calculated $\text{E1}/\mu\text{m}^2$
ER	19,400	15	1.8×10^6	93
Golgi	2,100	15	1.8×10^6	860
PM	3,400	—	—	—
Virus	0.008	—	180	22,000

The value used for the rate of synthesis of E1 was 1.2×10^5 mols/cell/min. Surface area of membranes is from Griffiths et al. (1984), protein dwell times from Green et al. (1980). Surface area of virus was calculated from data from Simons & Warren (1983) for capsid diameter and from Harrison et al. (1971) for membrane thickness. The value of 180 mols E1 is also from Simons & Warren (1983); this number is still in doubt, and some data suggest the figure may be as high as 240 (Bonsdorff & Harrison, 1975).

densities. Attempts to quantitate the E1 present by direct scanning of Coomassie stained gels were frustrated by overlapping peaks of endogenous protein, although the viral protein could be seen and was clearly more abundant in the Golgi fraction than in the ER. These gels also indicated that the plasma membrane fraction as prepared from infected cells was heavily contaminated with ER; this was supported with

TABLE III

Concentration of the Viral Protein E1 in Carbonate Washed Subcellular Fractions of Infected BHK Cells, Determined by Quantitative Immunoblotting

	Total	ER	Golgi
Protein		100	100
E1		0.19	1.48
Surface area of associated lipid		3.1	3.2
Density		70	515
E1/total protein (%)		0.14	1.14

Units of measure, protein, E1, micrograms; surface area, microns² $\times 10^{-10}$ (calculated); density, molecules/micron² lipid area.

cholesterol/phospholipid ratio, and data on this fraction has therefore been excluded.

DISCUSSION

In this paper, we have estimated the density of plasma membrane protein precursors in the membranes of the ER and Golgi complex. This was made possible by the use of the spike protein complex of SFV, which provides a relatively simple and well-defined model system for the biogenesis of plasma membrane proteins. These estimates involved determining the rate of synthesis of the spike proteins in infected BHK cells, and the density of endogenous proteins in the membranes of organelles isolated by subcellular fractionation.

The rate of synthesis of viral spike proteins in infected BHK cells was found to be 1.2×10^5 copies of each/cell/min. This figure is a mean of values obtained from two totally independent methods giving results in good agreement. The rate is high, but not abnormal even for a membrane protein (2), and is lower by an order of magnitude than the rate of albumin synthesis by hepatocytes (9).

The density of endogenous proteins found in the BHK cell membranes was between thirty and forty thousand molecules/ μm^2 . There are, however, three systematic errors in this estimate, which do not tend to cancel. These are (a) adventitious proteolysis of endogenous membrane protein during the cell fractionation; (b) incomplete removal of peripheral proteins by the carbonate washing procedure; and (c) the contribution the membrane proteins made to the surface area. All would tend to make the estimate high. For the last point, there are cases for which this contribution has been measured; the range of values obtained is from 10–25% of the membrane area in systems as diverse as the erythrocyte ghost (11), Sindbis virus (17) and the retinal rod outer disc (10). The densities found are in good agreement with estimates for other membranes obtained by a variety of techniques (3, 7, 23, 25). It is interesting to compare these figures with an extreme case such as cytochrome c oxidase crystals, which should indicate an upper limit for packing proteins into a lipid bilayer (12). From X-ray and electron microscopic data, the density of "cytochrome oxidase monomers" was determined to be $1.7 \times 10^4/\mu\text{m}^2$. This is the value one would expect to obtain by freeze-fracture and counting of intramembrane particles. Each "monomer", however, consists of 10 polypeptides, of which three are membrane-spanning. The density of integral membrane proteins is therefore $5.1 \times 10^4/\mu\text{m}^2$; the other proteins provide a good example of tightly bound, though strictly peripheral proteins. Despite the systematic errors, the correlation with results from other systems suggests that the esti-

mates are reasonably accurate.

The density of E1 in the intracellular membranes is extremely low, constituting only 0.14% of the endogenous ER protein. Since the other viral spike protein, p62, is present in an equimolar amount, the total plasma membrane protein precursor in the ER is 0.28% of the endogenous protein. What limits this density is unclear, but it is reasonable that, as suggested by Rothman (26), the levels of non-ER proteins should be kept low to prevent the ER assuming the characteristics of other compartments. There has been one previous estimate of the density of plasma membrane precursor proteins in the ER (26). A value of 0.01% of that found in the plasma membrane was obtained using data from liver. Our data, obtained by direct measurement as well as calculation, give a figure 28-fold higher. The difference may simply reflect the much larger amount of ER and longer turnover time of plasma membrane proteins in hepatocytes. It should be noted here that the close agreement between the calculated and measured densities of E1 in the ER and Golgi membranes is in itself good evidence that most, if not all, of the membrane measured as ER or Golgi in the morphometric study is involved in the synthesis and transport of viral membrane proteins, and that the isolated membrane fractions are representative of those compartments.

The viral protein is concentrated some 240-fold, from a density of 0.9×10^2 molecules/ μm^2 in the ER, to 2.2×10^4 molecules/ μm^2 in virus particles. A 9-fold concentration is found after transfer from the ER to the Golgi complex. These experiments, of course, provide only an average figure and any concentration gradient across the Golgi stack would not be observed. However, the available immunocytochemical data (e.g., 14, 15) show no such gradient, which suggests that concentration steps occur during transport to and from but not within the Golgi stack. If the transfer steps from Golgi to plasma membrane, and from plasma membrane to virus result in concentration of the same order of magnitude as that seen upon transfer from ER to Golgi, they would be more than sufficient to explain the densities observed. Data for the concentration that can be achieved at a single step are available only for the plasma membrane, where receptors are concentrated into coated pits. The best documented is the low-density lipoprotein receptor, which can be concentrated at least 100-fold in the plane of the membrane (1). The evidence that coated vesicles are involved in intracellular transport (27) makes it plausible that a mechanism of comparable selectivity is operating during transport from the ER and from the Golgi complex. The potential for concentration at just these two steps is clearly much higher than required.

It is not sufficient, however, to simply concentrate the proteins to be transported: they must also be purified from the endogenous proteins of the intracellular membranes that would otherwise necessarily contaminate them. Coated pits can not only concentrate selected proteins, but can also exclude others (5). The extent of exclusion, taking the raw data is between 10 and 100-fold. The data, obtained by immunoferritin binding to excluded antigens showed that labeling of coated pits was comparable with background labeling and the estimate is therefore a minimum figure. The product of the concentration and exclusion factors, 10^3 - 10^4 -fold, is the purification obtainable in a single step. It can also be argued that for vesicular traffic to occur but leave the membrane compartments in balance, an equal and opposite

traffic of vesicles is required, which returns material to the original donor membrane. This provides a further opportunity to collect any proteins that might have been transported in error; the purification achievable by a single such cycle would be in the range of 10^6 – 10^8 -fold. These figures imply that selection of proteins to be transported would, in a single step, result in contamination by endogenous proteins of, at most, 25%: a complete vesicle shuttle cycle would lower this to <0.03%, though it must be noted that the data for concentration and exclusion apply to the plasma membrane. Whether intracellular selection mechanisms are equally efficient, and what level of contamination is acceptable to the cells are not yet known.

The possible need for more than one purification step was first noted by Rothman (26), who proposed that purification would be best achieved by reselection from a partially purified pool of protein in a process analogous to fractional distillation. However, given that two steps are sufficient for purification, and that our results are generally applicable, purification could be achieved by a single vesicle shuttle cycle between two compartments, and would not require a stack of Golgi cisternae.

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