

# Biogenesis of the Rat Hepatocyte Plasma Membrane In Vivo: Comparison of the Pathways Taken by Apical and Basolateral Proteins Using Subcellular Fractionation

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**Abstract.** We have used pulse-chase metabolic radiolabeling with L-[<sup>35</sup>S]methionine in conjunction with subcellular fractionation and specific protein immunoprecipitation techniques to compare the posttranslational transport pathways taken by endogenous domain-specific integral proteins of the rat hepatocyte plasma membrane in vivo. Our results suggest that both apical (HA 4, dipeptidylpeptidase IV, and aminopeptidase N) and basolateral (CE 9 and the asialoglycoprotein receptor [ASGP-R]) proteins reach the hepatocyte plasma membrane with similar kinetics. The mature molecular mass form of each of these proteins reaches its maximum specific radioactivity in a purified hepatocyte plasma membrane fraction after only 45 min of chase. However, at this time, the mature radiolabeled apical proteins are not associated with vesicles derived from the apical domain of the hepatocyte plasma membrane, but instead are associated with vesicles which, by several criteria, appear to be basolateral plasma membrane. These vesicles: (a) fractionate like basolateral plasma membrane in sucrose density gradients and in free-flow electrophoresis; (b) can be separated from the bulk of the likely organellar contaminants, includ-

ing membranes derived from the late Golgi cisternae, transtubular network, and endosomes; (c) contain the proven basolateral constituents CE 9 and the ASGP-R, as judged by vesicle immunoadsorption using fixed *Staphylococcus aureus* cells and anti-ASGP-R antibodies; and (d) are oriented with their ectoplasmic surfaces facing outward, based on the results of vesicle immunoadsorption experiments using antibodies specific for the ectoplasmic domain of the ASGP-R. Only at times of chase > 45 min do significant amounts of the mature radiolabeled apical proteins arrive at the apical domain, and they do so at different rates. Approximate half-times for arrival are in the range of 90–120 min for aminopeptidase N and dipeptidylpeptidase IV whereas only 15–20% of the mature radiolabeled HA 4 associated with the hepatocyte plasma membrane fraction has become apical even after 150 min of chase. Our results suggest a mechanism for hepatocyte plasma membrane biogenesis in vivo in which all integral plasma membrane proteins are shipped first to the basolateral domain, followed by the specific retrieval and transport of apical proteins to the apical domain at distinct rates.

**T**HE mechanisms by which integral membrane proteins are sorted to distinct organellar locations have received considerable attention in contemporary cell biology. Our focus has been directed on one aspect of this central problem: how a polarized epithelial cell, the hepatocyte, can establish and maintain specialized plasma membrane domains with distinct protein compositions (for review see reference 39). We and others in this laboratory have used monoclonal and polyclonal antibodies to identify and characterize a number of integral proteins that are restricted to either the apical (bile canalicular) or the basolateral (sinusoidal/lateral) domains of the rat hepatocyte plasma membrane (2,

3, 9, 19, 36). In addition, we have established methods to prepare highly purified hepatocyte plasma membrane sheets from rat livers (18) and to separate apical and basolateral vesicles derived from these sheets (3). In this article we have used these and other subcellular fractionation techniques in conjunction with pulse-chase metabolic radiolabeling with L-[<sup>35</sup>S]methionine and immunoprecipitation of the domain-specific proteins to investigate the pathways taken by newly synthesized apical and basolateral proteins to their respective plasma membrane domains.

## Materials and Methods

### Labeling with L-[<sup>35</sup>S]Methionine

Young adult male Sprague-Dawley rats (125–200 g, CD strain; Charles River Breeding Labs, Wilmington, MA) were fasted for 20–24 h and then

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pulse-labeled by saphenous vein injection of 5 mCi of L-[<sup>35</sup>S]methionine (>800 Ci/mmol, SJ.204; Amersham Corp., Arlington Heights, IL) in 0.4 ml of PBS while under mild ethyl ether anaesthesia. 10 min later, a chase consisting of 2.5 ml of 20 mg/ml unlabeled L-methionine in PBS was administered by intraperitoneal injection, and additional chases (0.5 ml each) were supplied at 30-min intervals. After various periods of chase, the rats were killed by decapitation, and the livers were excised and perfused with ice-cold 0.9% saline in preparation for subcellular fractionation.

### Centrifugation in Sucrose Density Gradients

Hepatocyte plasma membrane sheets were prepared, vesiculated by sonication, and resolved in continuous sucrose density gradients (domain gradients, density range, 1.06–1.17 g/ml) as described (3, 18). Protease inhibitors (1 µg/ml of antipain and leupeptin, 1 mM phenylmethylsulfonyl fluoride [PMSF] and 100 KIU/ml of Trasylol) were included during homogenization of the liver tissue and sonication of the plasma membrane sheets. Typically, the vesicles derived from 3–4 mg of hepatocyte plasma membrane protein were loaded onto the large-scale 36-ml gradients (SW 28 rotor; Beckman Instruments, Inc., Fullerton, CA), and 12 3-ml fractions plus the pellet were collected. Liver microsomal (100K) fractions were prepared and resolved in continuous sucrose density gradients (microsomal gradients, density range, 1.10–1.25 g/ml) as described (8, 9). The protease inhibitors (see above) were included during homogenization of the liver tissue. Typically, the microsomes derived from 2.5–3 g of tissue (wet weight) were loaded onto 32-ml gradients, and 28 1.3-ml fractions were collected.

### Free-Flow Electrophoresis

Hepatocyte plasma membrane sheets were prepared in the presence of the protease inhibitors as described above (3, 18), except that the final 1,500-g, 10-min wash step was performed using chamber buffer (0.1 M mannitol, 0.2 mM Na<sub>4</sub>EDTA, 10 mM acetic acid, 10 mM triethanolamine, adjusted to pH 7.4 with NaOH) instead of 0.25 M sucrose. The membrane pellet obtained from a single rat liver was resuspended in 4 ml of chamber buffer and vesiculated for 45 s at setting 8 using a Polytron homogenizer (Brinkman Instruments Co., Westbury, NY) fitted with a 12-mm tip. The suspension was centrifuged for 10 min at 1,500 g, and the supernate was introduced into a free-flow apparatus (Elphor VAP 22; Bender-Hobein, Munich, Federal Republic of Germany) using a chamber buffer flow rate of 198 ml/h (setting 100). The chamber had been coated overnight with 3% wt/vol BSA in H<sub>2</sub>O and rinsed thoroughly with water and chamber buffer immediately before the run. The electrode buffer was: 2 mM Na<sub>4</sub>EDTA, 0.1 M acetic acid, and 0.1 M triethanolamine, adjusted to pH 7.4 with NaOH. The temperature (4°C) and the voltage (185 V/cm) were kept constant throughout the separation. 90 3.5-ml fractions were collected, adjacent fractions were pooled in groups of two (1 and 2, 3 and 4, etc.) and the fraction pools were renumbered, 1, 2, etc., starting from the anode.

### Immunoprecipitation and Immunoblotting

Immunoprecipitations were conducted under conditions that are known to give near quantitative recoveries as assessed by immunoblotting. Liver homogenates and hepatocyte plasma membrane sheets (at protein concentrations of 25–50 and 0.75–1.5 mg/ml, respectively) were extracted for 45 min at 4°C with 20 mM octyl-β-D-glucopyranoside, 0.5% wt/vol Triton X-100, 0.3 M NaCl, 0.025 M NaP<sub>i</sub>, pH 7.4, containing 0.02% wt/vol Na<sub>3</sub>N and the protease inhibitors (2, 16). The mixtures were centrifuged at 150,000 g for 60 min at 4°C. Aliquots (0.3 and 1 ml, respectively) of the supernates were incubated for 4–15 h at 4°C with 70 µl of the designated monoclonal antibody–Sepharose 4B slurry (1 mg of IgG/ml of slurry) in a final volume of 1 ml of the same buffer containing 0.5% wt/vol of BSA (2, 19). The beads were sedimented (13,000 g, 0.5 min) and washed six times, three times with the same buffer plus 0.5% wt/vol of albumin, twice with the same buffer without albumin, and once with 0.15 M NaCl, 0.02 M Tris-HCl, 0.02% wt/vol Na<sub>3</sub>N. To immunoprecipitate the proteins from the density gradient fractions, it was necessary to first concentrate the fractions by centrifugation at 150,000 g for 60 min after a sevenfold dilution in water containing the protease inhibitors. The equivalent of one-half of the detergent extract produced from each fraction was used in the procedure as described above. To immunoprecipitate the proteins from the free-flow electrophoresis fractions, it was necessary to first concentrate the fractions by centrifugation at 150,000 g for 60 min. The detergent extract produced from each 7.0-ml fraction (see above) was used in the immunoprecipitation procedure described above. The immunoprecipitations were often performed in a sequential fashion, with the supernate from the first immunoprecipitation being challenged

by a second monoclonal antibody–Sepharose conjugate and so on. The order in which the proteins were immunoprecipitated proved unimportant.

The immunoprecipitates were released from the washed beads by heating for 3 min at 100°C in the presence of 75 µl of gel sample buffer containing 55 mM Tris-HCl, 6 mM EDTA, and 4.5% wt/vol SDS, pH 8.9. The 13,000-g, 5-min supernates were reduced by the addition of 2.5 µl of 130 mg/ml dithiothreitol (DTT), heated for an additional 2 min, alkylated by the addition of 20 µl of 36 mg/ml iodoacetamide in 2 M sucrose, subjected to SDS-PAGE in 7.5% gels, and transferred to nitrocellulose as described (2, 3, 19). The blots were exposed to Kodak XAR-5 x-ray film for 2–6 wk at –70°C. The amount of radioactivity in the individual protein bands was determined by laser densitometric analysis (Biomed Instruments, Inc., Fullerton, CA) of the corresponding autoradiograms. For each of the proteins examined, it was possible to resolve the mature molecular mass (terminally glycosylated; see reference 2) forms of the proteins from their corresponding immature (probably high mannose) precursors. The latter are readily detected when the proteins are immunoprecipitated from liver homogenates immediately after pulse labeling or after short periods of chase. Only the mature forms of the proteins were considered in the experiments described here. The chemical amounts of the rat hepatocyte plasma membrane proteins and α-2,6-sialyltransferase in the different fractions were compared by quantitative immunoblotting using affinity-purified rabbit polyclonal antibodies and <sup>125</sup>I-protein A as described (1–3, 19, 32). For each of the proteins analyzed, we verified that the autoradiographic response was linear over a range of fraction inputs. Antibodies directed against the rat liver α-2,6-sialyltransferase were affinity-purified from the corresponding rabbit antiserum on columns of sialyltransferase Sepharose using isotonic glycine, pH 3.0, as eluant (2, 9, 32). Both the antiserum and purified enzyme were generously supplied by Dr. Gerald Hart (Dept. of Biological Chemistry, Johns Hopkins University School of Medicine) (46). For anti-sialyltransferase immunoblotting, the gel samples were prepared by heating at 50°C for 10 min under nonreducing conditions (9). In the case of the experiment shown in Fig. 1, the <sup>35</sup>S incorporation data were normalized to the chemical amounts of the proteins immunoprecipitated from the hepatocyte plasma membrane sheets prepared at each time point as determined by immunoblotting. The percent apical values plotted in Fig. 4 were derived from a comparison of the distributions of the mature radiolabeled apical proteins (Figs. 2 B and 3, A and B) with those of the steady-state domain markers, HA 4 and CE 9 (Fig. 2 C) in the apical region (fraction 5) and the basolateral region (fraction 9) of the domain gradient. Percent apical =  $100a/(a + b)$ , where  $a = ([\% \text{ of radiolabeled protein in fraction 5}] - [\% \text{ of CE 9 in fraction 5}]) / ([\% \text{ of HA 4 in fraction 5}] - [\% \text{ of CE 9 in fraction 5}])$ , and  $b = ([\% \text{ of radiolabeled protein in fraction 9}] - [\% \text{ of HA 4 in fraction 9}]) / ([\% \text{ of CE 9 in fraction 9}] - [\% \text{ of HA 4 in fraction 9}])$ .

### Vesicle Immunoadsorption

Vesicle immunoadsorptions were performed with fixed *Staphylococcus aureus* cells exactly as described previously (20, 32). 50-µl aliquots of pooled fractions 13–16 (the plasma membrane region) from microsomal gradients prepared from L-[<sup>35</sup>S]methionine-labeled rat livers after 45 min of chase were mixed with 3 µg of the designated rabbit preimmune IgG or anti-asialoglycoprotein receptor (ASGP-R)<sup>1</sup> antibody. To obtain a significant <sup>35</sup>S autoradiographic signal, it was necessary to perform 20 replicate immunoadsorptions with each antibody type and pool the resultant *S. aureus* pellets. The combined pellets were detergent extracted in a final volume of 3 ml and centrifuged at 150,000 g for 60 min as described above. The proteins were immunoprecipitated from the supernates and analyzed by SDS-PAGE and autoradiography as described above. Aliquots of the pooled *S. aureus* pellets were also analyzed directly by immunoblotting as described (1, 2, 19, 32).

The preparation and characterization of the affinity-purified anti-total rabbit polyclonal anti-ASGP-R antibody, which reacts with both the ectoplasmic and the cytoplasmic domains of the ASGP-R in vesicle immunoadsorption experiments, has been described previously (32). The anti-head anti-ASGP-R antibody was prepared and affinity purified in an identical fashion, except that a proteolytic fragment of the ASGP-R served as the immunogen and the affinity adsorbent. This fragment was generated by limited papain digestion of the vesicles present in the first 1,500-g, 10-min supernate (fraction 4) from our hepatocyte plasma membrane sheet preparation procedure (18). This fraction contains the majority of the latent (presumably endosomal) ASGP-R present in rat liver homogenates and is substantially depleted of hepatocyte plasma membrane, which contains the nonlatent

1. **Abbreviations used in this paper:** AP, aminopeptidase N; ASGP-R, asialoglycoprotein receptor; DPP IV, dipeptidylpeptidase IV.

receptor (18, 45). Fraction 4, at a protein concentration of 10 mg/ml, was treated for 1 h at 10°C with 0.2 mg/ml of papain in 50 mM NaPi, 5 mM L-cysteine, 0.03 mM DTT, 0.25 M sucrose, pH 7.3. The papain had been activated by preincubation at 2 mg/ml in this same buffer at pH 6.4 for 30 min at 10°C. The reaction was stopped by the addition of iodoacetamide (10 mM), antipain (5 µg/ml), and leupeptin (5 µg/ml). By immunoblotting using the anti-total ASGP-R antibody (32), the major 42.5-kD ASGP-R subunit (RHL-1, reference 7) was nearly quantitatively converted to a 37-kD species as a result of this treatment. The membrane vesicles were collected by centrifugation (100,000 g, 60 min), extracted with Triton X-100, and the ASGP-R fragment isolated by affinity chromatography on asialo-orosomucoid-Sepharose as described previously for the intact receptor (32). Unlike the intact ASGP-R, the purified fragment did not react on immunoblots with an antibody that we raised to a tuberculin-purified protein derivative conjugate of the cytoplasmically disposed NH<sub>2</sub>-terminal decapeptide of the ASGP-R.

### Miscellaneous Procedures

For measurement of galactosyl transferase activity, 10- to 50-µl aliquots of the various membrane fractions were incubated for 1 h at 37°C in a final volume of 100 µl containing 22 mM Hepes-NaOH (pH 7.0), 25 mM 2-mercaptoethanol, 0.18% wt/vol Triton X-100, 18 mM MnCl<sub>2</sub>, and 0.45 mM (3 × 10<sup>6</sup> cpm/ml) uridine diphospho-D-[6-<sup>3</sup>H]galactose (TRK.513; Amersham Corp., Arlington Heights, IL) plus or minus exogenous substrate (7.5 mg/ml Trypsin inhibitor, type III-O; Sigma Chemical Co., St. Louis, MO). The reaction was stopped by the addition of 1 ml of 10% wt/vol TCA, and the precipitate was collected and washed under vacuum on 25-mm diameter filters (type HA, 0.45-µm pore; Millipore Corp., Bedford, MA). The filters were placed in 20-ml glass scintillation vials and incubated with 1 ml of 1 N HCl for 20 min at 80°C. 1 ml of ethyl acetate was added, and the vials were shaken overnight at room temperature to allow the filters to dissolve before adding 10 ml of Aquasol 2 (New England Nuclear, Boston, MA) and counting in the tritium channel of a scintillation spectrometer (LS 7000; Beckman Instruments).

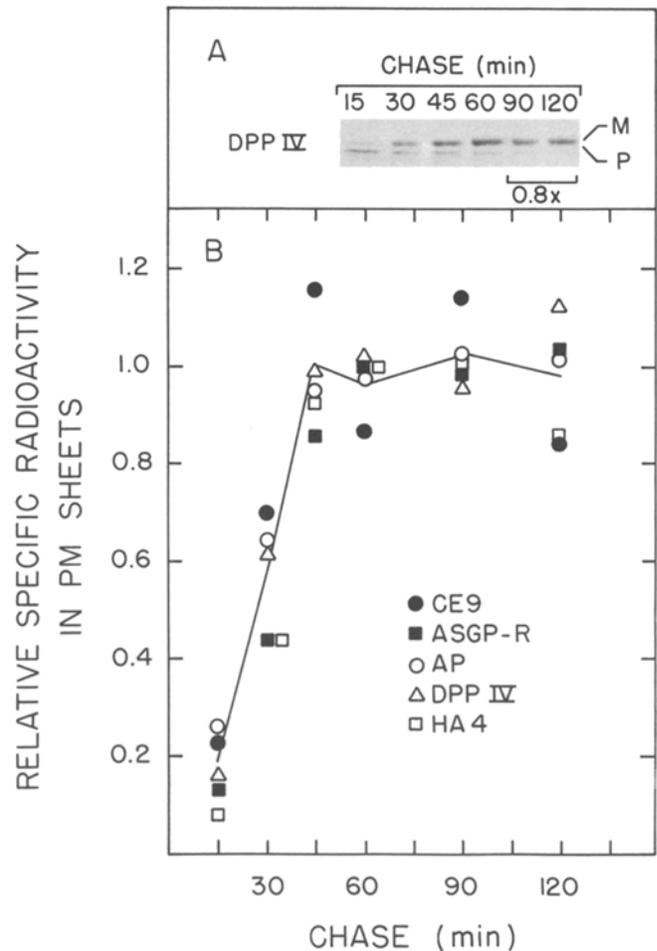
To determine the distribution of <sup>125</sup>I-asialo-orosomucoid-labeled endosomes in the domain gradients, hepatocyte plasma membrane sheets were prepared as usual (18) from an isolated perfused rat liver that had been allowed to continuously endocytose <sup>125</sup>I-asialo-orosomucoid for 14 min at 37°C (32). The recoveries and domain gradient distributions of HA 4 and CE 9 were not significantly altered by the perfusion procedure (see also reference 3). With this kind of labeling procedure, the <sup>125</sup>I-asialo-orosomucoid serves as a marker for both peripheral and internal (Golgi-lysosome region) endosomes (45). That portion of the <sup>125</sup>I-asialo-orosomucoid recovered in the plasma membrane sheet fraction that was released from the vesicles during sonication and, therefore, remained in the loading zone (fractions 1 and 2) of the domain gradient amounted to ~25% and was not included in the distributional analysis shown in Fig. 5.

Alkaline phosphodiesterase I, an enzyme marker for the hepatocyte plasma membrane, and NADH cytochrome c reductase, an enzyme marker for the endoplasmic reticulum, were assayed as described previously (18).

## Results

### Appearance of the Newly Synthesized Proteins in the Hepatocyte Plasma Membrane Sheet Fraction

The first group of experiments was performed to determine the rates at which the newly synthesized domain-specific proteins arrived at the hepatocyte plasma membrane. Three apical proteins, aminopeptidase N (AP), dipeptidylpeptidase IV (DPP IV), and HA 4, and two basolateral proteins, CE 9 and the ASGP-R, were immunoprecipitated from detergent extracts of rat hepatocyte plasma membrane sheets isolated at different times of chase after a 10-min pulse-labeling with L-[<sup>35</sup>S]methionine. The immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and subjected to autoradiography. Densitometric analysis of the autoradiograms (Fig. 1 A) suggested that all five proteins examined, apical and basolateral alike, reached the plasma membrane at approximately the same rate (Fig. 1 B). The specific radio-



**Figure 1.** Kinetics of reaching the hepatocyte plasma membrane sheet fraction. Rats were pulse-labeled for 10 min with L-[<sup>35</sup>S]methionine and then chased with unlabeled L-methionine for the designated time interval. The proteins were immunoprecipitated from the hepatocyte plasma membrane sheet fractions, and the levels of <sup>35</sup>S radioactivity in the mature forms of each were determined by blot autoradiography. (A) Autoradiogram of blot of DPP IV immunoprecipitates. M, mature; P, precursor. The load for the 90 and 120 min lanes was 80% of that loaded for the other time points. (B) Graph of the <sup>35</sup>S-labeling data obtained for all five of the proteins examined after normalization to the chemical amounts of protein immunoprecipitated from each hepatocyte plasma membrane sheet fraction (as determined by immunoblotting). A single liver was analyzed for each time of chase. Each point represents the average of duplicate protein immunoprecipitations. PM, plasma membrane.

activities of the mature molecular mass forms of these proteins in the plasma membrane fraction increased dramatically during the first part of the chase period and then leveled off at their maxima beginning at 45 min. The presence of detectable levels of radiolabeled precursor forms of these proteins in these immunoprecipitates, particularly after short chase times (e.g., see DPP IV precursor band marked by P in Fig. 1 A), is indicative of the level of endoplasmic reticulum contamination in the hepatocyte plasma membrane sheet fraction (reference 18; cf. recoveries of NADH cytochrome c reductase and alkaline phosphodiesterase I in Table I).

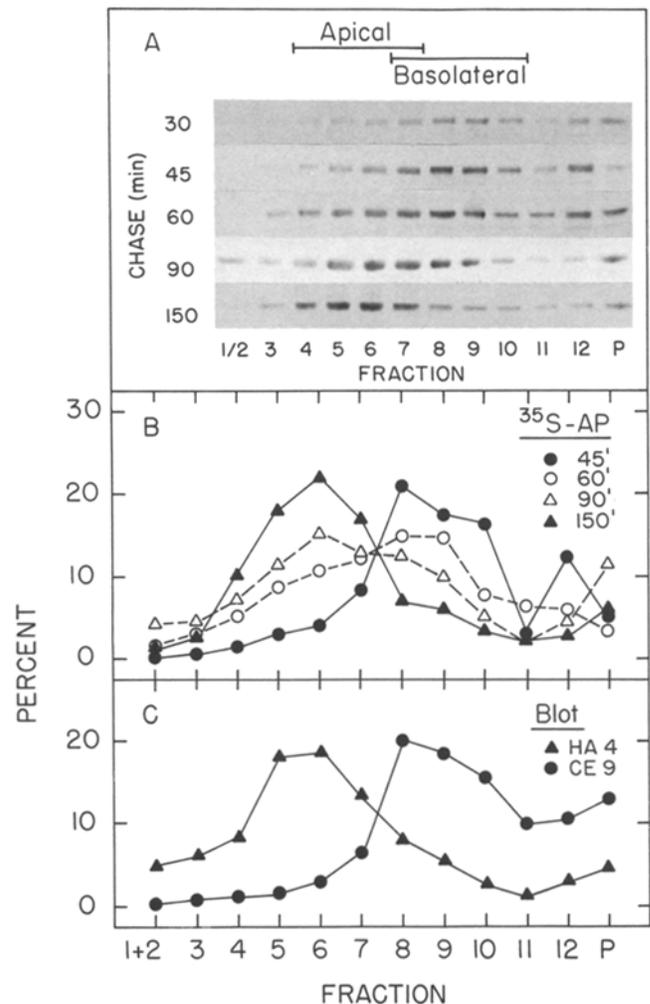
**Table I. Yields of Mature  $^{35}\text{S}$ -labeled Proteins and Other Markers in the Hepatocyte Plasma Membrane Sheet Preparation**

Marker	Yield
	%
NADH-cytochrome <i>c</i> reductase	0.5
Endosomes	0.5
Sialyltransferase	0.7
Galactosyltransferase	1.0
Alkaline phosphodiesterase I	16
Immunoblot	
CE 9	8.5
ASGP-R	1.3
HA 4	11
DPP IV	11
AP	6.8
$^{35}\text{S}$ (mature)	
CE 9	5.2
ASGP-R	2.0
HA 4	6.8
DPP IV	5.7
AP	8.0

Hepatocyte plasma membrane sheets were prepared from rat liver that had been pulse-labeled with L- $^{35}\text{S}$ methionine for 10 min and then chased for 45 min. The filtered homogenate and plasma membrane sheet fractions were assayed for the following: sialyltransferase and the plasma membrane proteins by immunoblotting; NADH-cytochrome *c* reductase, galactosyltransferase, and alkaline phosphodiesterase I by enzyme assay; and mature  $^{35}\text{S}$ -labeled proteins by immunoprecipitation as described in Materials and Methods. To estimate the yield of endosomes in a plasma membrane sheet fraction, a second liver was exposed to  $^{125}\text{I}$ -asialo-orosomucoid for 14 min at 37°C in isolated perfusion, and the amounts of sedimentable (150,000 g, 60 min)  $^{125}\text{I}$  radioactivity in the filtered homogenate and plasma membrane sheet fractions were determined. The yield of alkaline phosphodiesterase I is typical of that of other plasma membrane marker enzymes; all are obtained with a 10–20% yield and show a 40–60-fold enrichment (3, 18).

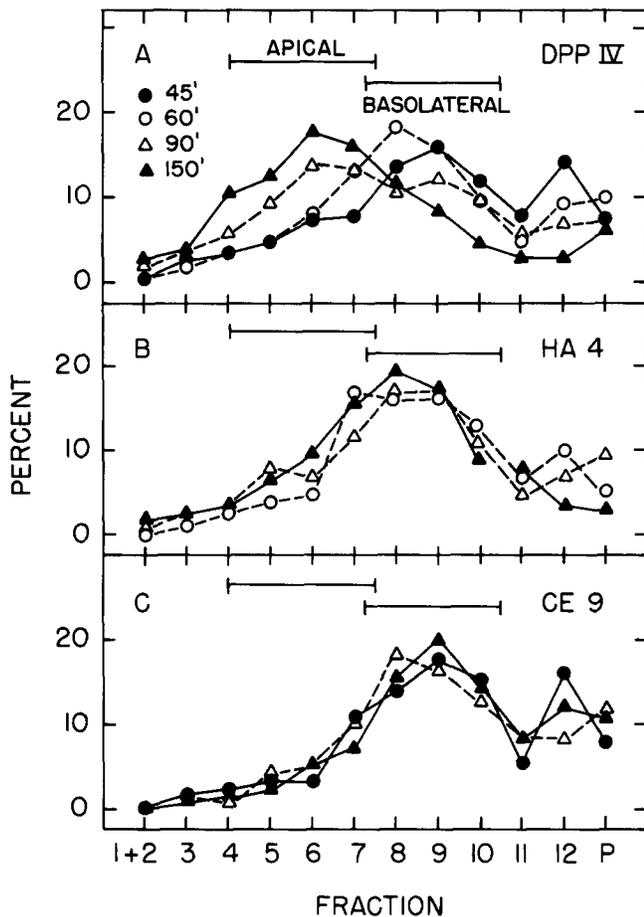
### Domain Gradient Analysis at Different Times of Chase

We next addressed the question of domain localization for the newly synthesized plasma membrane proteins. At selected times of chase the hepatocyte plasma membrane sheets were first resolved into apical and basolateral plasma membrane vesicles in sucrose density gradients, and the proteins were immunoprecipitated from detergent extracts of the gradient fractions. Much to our surprise, the distribution of the mature radiolabeled apical proteins in these gradients was found to change as a function of the chase time. This phenomenon is illustrated for AP in Fig. 2. At the early times of chase (30 and 45 min), mature radiolabeled AP did not distribute with the apical domain marker (HA 4), but instead exhibited a distribution that was almost identical to that of the basolateral domain marker CE 9. With increasing time of chase, the distribution of the mature radiolabeled AP was shifted progressively toward the lighter (apical) region of the gradients, so that only after 150 min did it become coincident with that of the apical domain marker HA 4. At intermediate chase times (60 and 90 min), the observed distribution appeared to represent some weighted average of apical- and basolateral-like distributions. Thus, at the time when mature AP first reached its maximum specific radioactivity in the plasma membrane fraction (45 min), it was not present in apical plasma membrane vesicles, but in vesicles that fractionated more like those derived from the basolateral domain. The mature forms of the other two apical proteins ex-



**Figure 2.** Domain localization of the newly synthesized mature AP. Hepatocyte plasma membrane sheets were isolated from rats after a 10-min pulse-labeling with L- $^{35}\text{S}$ methionine and the designated period of chase. They were vesiculated by sonication and resolved into apical and basolateral populations in sucrose density gradients. The apical protein AP was immunoprecipitated from the gradient fractions, and the distribution of the mature radiolabeled AP was determined by blot autoradiography. (A) Autoradiogram of the blots of the AP immunoprecipitates. Little, if any, of AP's lower molecular mass precursor can be seen. The brackets define the positions of apical and basolateral domain markers (see C). (B) Graph of the data obtained from the densitometric analysis of the autoradiograms shown in A, with the distribution of the mature radiolabeled AP plotted as a percentage of that recovered in the gradient for each time of chase. ', min. (C) Distributions of the apical protein HA 4 and the basolateral protein CE 9 in these gradients were determined by immunoblotting and plotted as a percentage of the recovered antigen.

amined, DPP IV and HA 4, were also found to distribute in the basolateral region of the density gradients at the early times of chase (Fig. 3, A and B). We used relative peak heights for the three radiolabeled apical proteins in gradient fractions 5 and 9 to quantify the percentages of the newly synthesized proteins in the apical and basolateral regions of the gradients, respectively, after different times of chase (see Materials and Methods). Fig. 4 reveals that the proteins

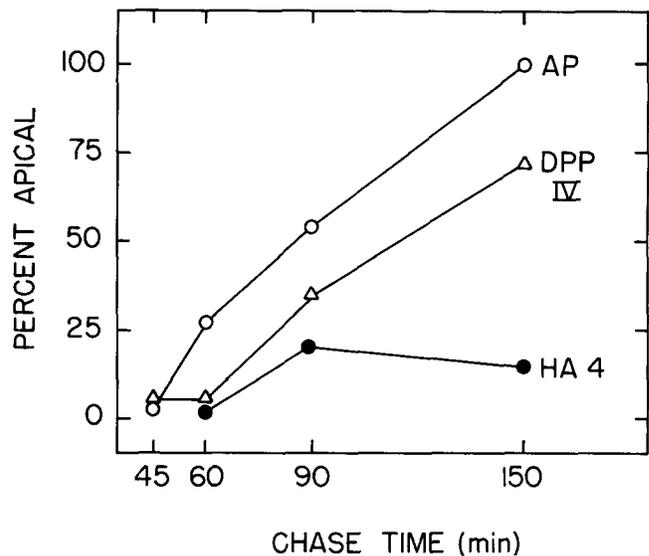


**Figure 3.** Domain localization for the other newly synthesized proteins. The distributions of the mature forms of the radiolabeled DPP IV (A), HA 4 (B), and CE 9 (C) in the domain gradients after the different times of chase were determined and plotted as described for AP in Fig. 2.

moved into the apical region of the gradients at different rates. AP was the fastest, with 50% of the mature radiolabeled form distributed apically after ~90 min and 100% after ~150 min of chase. The percentage of the mature radiolabeled DPP IV that was apical increased roughly in parallel with AP but appeared to lag 15–30 min behind AP over the whole time course. We have not yet examined enough animals to know if this difference between AP and DPP IV is statistically significant. Perhaps the strangest behavior was exhibited by HA 4, which appeared to reach a plateau with only 15–20% of the mature radiolabeled protein in the apical region, even after 150 min of chase. In comparison, the basolateral protein CE 9 was observed in the basolateral region of the gradients at all times of chase (Fig. 3 C).

#### Possible Involvement of Organellar Contaminants

The next group of experiments was undertaken to determine whether the membrane containing the mature radiolabeled apical proteins at 45 min of chase represented bona fide basolateral plasma membrane or some organellar contaminant(s) in the plasma membrane sheet preparation, which, coincidentally, fractionated like basolateral plasma membrane



**Figure 4.** Kinetics of reaching the apical domain. The percentages of the radiolabeled mature forms of AP, DPP, and HA 4 that were distributed apically in the domain gradients at different times of chase were calculated as described in Materials and Methods from the data shown in Figs. 2 B and 3, A and B, respectively.

in the domain gradients. Since we were monitoring the mature molecular mass (terminally glycosylated) forms of the newly synthesized proteins, contamination by the endoplasmic reticulum was not a concern. The most likely contaminants were thought to derive from the late Golgi cisternae and beyond in the pathway. As a result, we focused our attention on the fractionation behavior of markers of the late Golgi cisternae (galactosyl- and sialyltransferases; references 37, 38, 41), the transtubular network (sialyltransferase, reference 38), and the endosomes (labeled by  $^{125}\text{I}$ -asialo-orosomucoid; references 32 and 45), the latter because they are thought to be involved in the shuttling of hepatocyte plasma membrane proteins both between organelles (e.g., between the plasma membrane and lysosomes; references 8 and 9) and between different plasma membrane domains (e.g., between basolateral and apical domains in the transcytosis of the polymeric immunoglobulin A receptor complex; references 16, 31, 33, 40, 43). Table I lists the recoveries of these various organellar markers in the hepatocyte plasma membrane sheet fraction and compares them with those of a representative hepatocyte plasma membrane marker enzyme (alkaline phosphodiesterase I, see references 3 and 18), the individual domain-specific proteins themselves (HA 4, CE 9, etc.; see references 1, 3, 18, 19, 36) and the mature forms of the radiolabeled plasma membrane proteins at 45 min of chase. The recoveries of the mature radiolabeled CE 9, HA 4, DPP IV, and AP (5.2–8.0%) were considerably greater than those of the organellar contaminants (0.5–1%) and more similar to those of the unlabeled plasma membrane proteins as determined by immunoblotting (6.8–11%). The lower recovery of the ASGP-R (whether radiolabeled or unlabeled), compared with those of the other plasma membrane proteins examined, was consistent with the fact that as much as 80% of this receptor is believed to be located intracellularly in endosomes (18, 19). A similar explanation could account for why the recoveries of some of the other

plasma membrane proteins as determined by immunoblotting were less than that of the representative marker enzyme alkaline phosphodiesterase I (3, 18). All three of the apical proteins were found in low density membrane vesicles when microsomal density gradients were analyzed by immunoblotting (see below and Fig. 7). In principle, these vesicles could be derived from intracellular organelles or perhaps from other cell types (see below).

The glycosyltransferases, but not the endosomes, recovered in the hepatocyte plasma membrane sheet preparation (Table I) were found to distribute with the basolateral domain marker CE 9, and hence with the mature radiolabeled apical proteins, in the domain gradients (Fig. 5). However, we were able to achieve a partial separation of these glycosyltransferase-positive vesicles from the basolateral plasma membrane vesicles using the technique of free-flow electrophoresis (Fig. 6 B), which fractionates vesicles according to their surface charge density. With this method, the apical plasma membrane vesicles (marked by HA 4 in Fig. 6 B) exhibited the most dramatic anodic migration, with the sialyltransferase-containing vesicles (ST in Fig. 6 B) migrating the least. In fact, the latter were found to be concentrated in a relatively sharp peak, which also contained the bulk of the galactosyltransferase activity recovered in the sheet preparation (not shown). The migration of the basolateral plasma membrane vesicles (marked by CE 9 in Fig. 6 B) was intermediate, thus allowing their partial resolution from both the apical vesicles and the glycosyltransferase-positive vesicles. The distributions of the mature form of one representative newly synthesized apical protein, DPP IV, and of the newly synthesized basolateral protein CE 9 at 45 min of chase are shown in Fig. 6 A. Note that the distributions of the mature radiolabeled DPP IV and CE 9 were very similar and were virtually indistinguishable from that of the steady-state basolateral marker CE 9 (broken line in Fig. 6 A). Thus, at 45 min of chase, the mature forms of the radiolabeled apical proteins were present in vesicles that fractionated like basolateral plasma membrane using two quite independent separation techniques, density gradient centrifugation and free-flow electrophoresis. Furthermore, they could be distin-

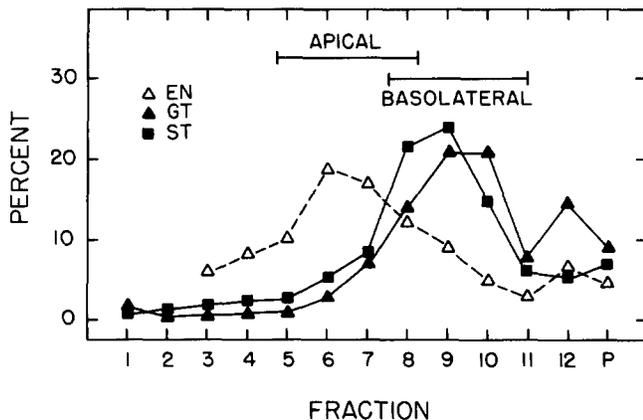


Figure 5. Distributions of contaminating organelles in the domain gradients. The distributions of  $^{125}\text{I}$ -asialo-orosomucoid-labeled endosomes (EN), galactosyltransferase (GT), and sialyltransferase (ST) in the domain gradients are plotted as a percentage of the recovered marker.

guished from the bulk of the likely organellar contaminants, including those derived from endosomes, the late Golgi cisternae, and the transtubular network.

We also examined the distributions of the various markers in a different sucrose density gradient system using a much cruder rat liver microsomal fraction (Fig. 7). Although apical and basolateral vesicles were not resolved in this gradient, the hepatocyte plasma membrane vesicles (marked by the immunoblot profile for CE 9 in Fig. 7 A, open circles) were separated from the majority of the glycosyltransferase-positive vesicles (Fig. 7 A: sialyltransferase, solid squares; galactosyltransferase, solid triangles), and endosomes (see also references 9 and 45). The latter fractionate like galactosyltransferase in this gradient (9, 45). Note that the immunoblot profiles for the apical proteins (Fig. 7, B-D, open circles) showed that considerable amounts of the proteins were also present in the lower density Golgi/endosome region of the gradient (between fraction pools 3 and 4 and 9 and 10). This trend, which was most noticeable for DPP IV (Fig. 7 B) and AP (Fig. 7 C), might indicate that these apical pro-

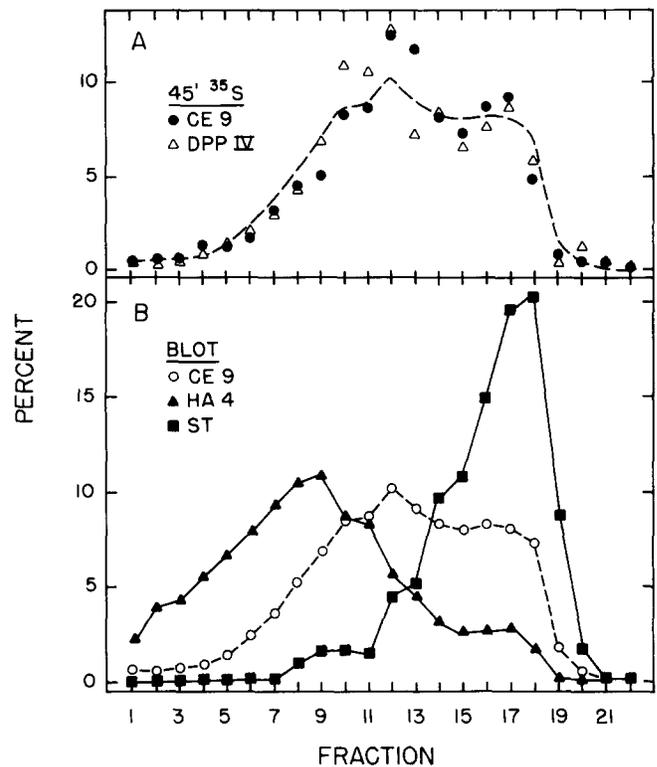
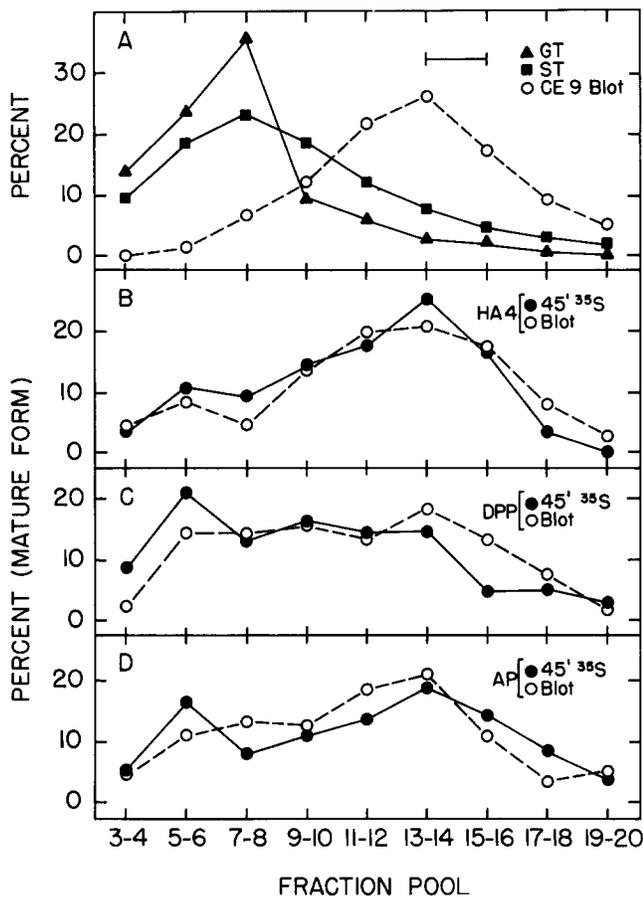


Figure 6. Free-flow electrophoretic analysis of the hepatocyte plasma membrane sheet fraction after 45 min of chase. Hepatocyte plasma membrane sheets were isolated from pulse-labeled rats at 45 min of chase, vesiculated by Polytron homogenization, and resolved by free-flow electrophoresis. (Fraction 1 is nearest the anode.) The apical protein DPP IV and the basolateral protein CE 9 were immunoprecipitated from the fractions. (A) The distributions of the mature radiolabeled DPP IV and CE 9 were determined by blot autoradiography and plotted as a percentage of that recovered in the experiment. The broken line shows the steady-state distribution of CE 9 as determined by immunoblotting (replotted in B). (B) The distributions of the apical protein HA 4, the basolateral protein CE 9, and sialyltransferase were determined by immunoblotting and plotted as a percentage of the recovered antigen.



**Figure 7.** Microsomal gradient analysis of the mature radiolabeled apical proteins at 45 min of chase. Liver microsomal fractions were isolated from pulse-labeled rats at 45 min of chase and were resolved in sucrose density gradients. The apical proteins HA 4 (B), DPP IV (C), and AP (D) were immunoprecipitated from the gradient fractions, and the distributions of the radiolabeled mature forms of the proteins (solid circles) were determined by blot autoradiography and plotted as a percentage of that recovered in the gradient. The steady-state distributions of these proteins in this gradient, as determined by immunoblotting, are also given (open circles in B, C, and D). The distributions of galactosyltransferase (GT), sialyltransferase (ST), and the plasma membrane marker CE 9 in this gradient are shown in A. The bar in A designates the fraction pool that was used in the vesicle immunoadsorption experiments (see Table II).

teins have intracellular pools in the steady state. However, we have never detected intracellular pools of these proteins in any of our light or electron microscopic immunolocalization experiments (references 1, 19, 36; and unpublished data). An alternative explanation is that the low density vesicles containing the apical proteins are derived from the plasma membrane of the other cell types in the liver. We have preliminary evidence that DPP IV may be present at the surface of the endothelial cells (unpublished data). Plasma membrane derived from the nonhepatocyte cell types does not significantly contaminate the hepatocyte plasma sheets (3).

As expected from the results shown in Fig. 1, the distributions of the mature forms of the radiolabeled apical proteins at 45 min of chase were found to be similar to their steady-state distributions in the microsomal gradient as determined by immunoblotting (cf. open and solid symbols in Fig. 7,

B-D). Most importantly, a significant fraction of each (45–65%) was present in the plasma membrane region of this gradient (between fraction pools 9 and 10 and 19 and 20). This range of percentages was similar to that which could be calculated from the  $^{35}\text{S}$ -protein recovery data in Table I after correction by a multiplicative factor of about six to normalize the recovery of the plasma membrane marker enzyme alkaline phosphodiesterase I in the sheet preparation to 100%. Thus, by either estimation, a substantial percentage of the mature radiolabeled proteins at 45 min of chase was detected in the hepatocyte plasma membrane. Furthermore, Fig. 7 illustrates that, even when analyzing the crude rat liver microsomal fractions, a large proportion of the vesicles containing the mature radiolabeled proteins at 45 min of chase (Fig. 7, B-D) could be separated from the cellular glycosyltransferase-positive vesicles and endosomes.

### Vesicle Immunoadsorption

We next used vesicle immunoadsorption with antibodies directed against a basolateral protein, the ASGP-R, to ask questions regarding the molecular composition and disposition of those vesicles containing the radiolabeled plasma membrane proteins at 45 min of chase. This procedure has been worked out in detail in our laboratory (20, 32) for the isolation of ASGP-R-positive endosomes from rat liver microsomal fractions. With this procedure, the membrane vesicles are incubated sequentially with affinity-purified rabbit anti-receptor antibodies followed by fixed *S. aureus* cells, the mixture is diluted, and the bacteria with bound antibody-vesicle complexes are collected by low speed centrifugation. For our experiments we used the plasma membrane peak (fraction pool 13–16, bar in Fig. 7 A) derived from liver microsomal gradients after 45 min of chase and two anti-ASGP-R antibodies. One antibody, called anti-total, was directed against the entire receptor molecule (both cytoplasmic and ectoplasmic domains). The other, called anti-head, was directed exclusively against the ectoplasmic domain of the protein (see Materials and Methods). When compared in the standard immunoadsorption assay using 3  $\mu\text{g}$  of affinity-purified antibody and 100  $\mu\text{g}$  of microsomal protein from a liver that had been allowed to endocytose  $^{125}\text{I}$ -asialo-orosomucoid for 14 min during isolated perfusion at 37°C (32), the anti-total antibody immunoadsorbed eight times more of the  $^{125}\text{I}$ -asialo-orosomucoid-containing endosomes than the anti-head antibody (57% vs. 7%). Despite this difference, these two antibodies could immunoadsorb similar amounts of the basolateral plasma membrane vesicles when tested against the vesicles in the plasma membrane region (pool of fractions 13–16) of the microsomal gradients (see Table II). The vesicles derived from the hepatocyte plasma membrane sheets could not be used in the immunoadsorption experiments because they consistently gave unacceptably high levels of nonspecific immunoadsorption. Two criteria by which we could justify the use of this alternate plasma membrane preparation were: (a) at 45 min of chase, the recoveries of the mature radiolabeled proteins in the two preparations were quite similar (see paragraph above); and (b) both preparations showed a similar extent of contamination by sialyltransferase. The sialyltransferase/CE 9 ratio in fraction pool 13–16 of the microsomal gradient was found to be only one to two times greater than that in the hepatocyte plasma membrane sheets.

**Table II. Immunoadsorption of <sup>35</sup>S-labeled Plasma Membrane Vesicles with Anti-total and Anti-head ASGP-R Antibodies**

Domain localization	Protein	Immunoadsorbed				Ratio of <sup>35</sup> S/immunoblot	
		<sup>35</sup> S (mature)		Immunoblot		Anti-total	Anti-head
		Anti-total	Anti-head	Anti-total	Anti-head		
		% of added	% of added	% of added	% of added		
Basolateral	ASGP-R	ND*	ND	66	65	—	—
	CE 9	59	63	76	67	0.78	0.94
Apical	DPP IV	61	65	11	10	5.5	6.5
	HA 4	46	52	10	13	4.6	4.0
	AP	35	37	9	9	3.9	4.1

Microsomal gradients were prepared from a rat that had been pulse-labeled with L-[<sup>35</sup>S]methionine for 10 min and then chased for 45 min. The vesicles from the plasma membrane region (fraction pool 13–16; see bar in Fig. 7 A) were subjected to immunoadsorption using anti-total or anti-head ASGP-R antibodies and *S. aureus* cells as described in Materials and Methods. The percentages of the added <sup>35</sup>S-labeled proteins (mature) and unlabeled proteins that were immunoadsorbed were determined by immunoprecipitation and immunoblotting, respectively, and have been corrected for the level of nonspecific immunoadsorption (~10%) observed using pre- or nonimmune antibodies.

\* ND, not determined because of poor detergent solubilization from the anti-ASGP-R immunoadsorbent in preparation for immunoprecipitation.

For analysis of the <sup>35</sup>S-labeled plasma membrane proteins, it was necessary to perform the immunoadsorption experiment on a much larger scale; the bacterial pellets from 20 individual immunoadsorption tubes were pooled, extracted with detergent, and the individual radiolabeled proteins were isolated by immunoprecipitation. Table II lists the immunoadsorption efficiencies for the mature forms of the different radiolabeled plasma membrane proteins at 45 min of chase and compares them with the values for the chemical amounts of these proteins immunoadsorbed as determined by immunoblotting. Each entry represents the percentage of the added protein that was immunoadsorbed. Whether using anti-total or anti-head antibodies: (a) the chemical amounts of the basolateral proteins that were immunoadsorbed were six to seven times greater than the chemical amounts of the apical proteins that were immunoadsorbed (66–76% vs. 9–11%), indicating that the immunoadsorption was indeed domain-specific; and (b) the percentages of the mature radiolabeled apical proteins that were immunoadsorbed were four to six times greater than the chemical amounts of the apical proteins that were immunoadsorbed (35–61% vs. 9–11%). In fact, the immunoadsorption efficiency for one radiolabeled apical protein, DPP IV, even approached that observed for the unlabeled and <sup>35</sup>S-labeled basolateral proteins. These results indicated that, at 45 min of chase, the newly synthesized apical proteins were substantially enriched (four- to sixfold) over their unlabeled counterparts in vesicles containing a basolateral protein, the ASGP-R. That the immunoadsorption results were virtually identical using the anti-head and anti-total antibodies suggested that these vesicles obtained from the plasma membrane region of the microsomal gradient were predominantly oriented with their ectoplasmic domains facing outward. Based upon the results of ligand binding studies performed in the presence and absence of permeabilizing detergents (8, 9, 45), the plasma membrane vesicles obtained from the microsomal gradients are known to be preferentially oriented ectoplasmic-side-out, whereas those vesicles derived from intracellular organelles, such as endosomes, have the opposite orientation.

## Discussion

We have examined hepatocyte plasma membrane biogenesis in vivo using pulse-chase metabolic radiolabeling in con-

junction with subcellular fractionation and immunoprecipitation techniques. The posttranslational transport pathways taken by endogenous apical and basolateral proteins were compared in an attempt to reveal the site where these proteins are sorted in this polarized epithelial cell.

### *Apical and Basolateral Proteins Reach the Hepatocyte Plasma Membrane Rapidly and with Similar Kinetics*

Our results suggest that both the apical and basolateral proteins reach the hepatocyte plasma membrane rapidly and with similar kinetics. The three apical and two basolateral proteins examined each reach their maximum specific radioactivity in a highly purified hepatocyte plasma membrane sheet fraction after only 45 min of chase (Fig. 1). An earlier study on the biosynthesis of two rat hepatocyte plasma membrane proteins, DPP IV and nucleotide pyrophosphatase, reported that almost 2 h were required for these two proteins to reach their maximum specific radioactivities in a plasma membrane fraction (10). The apparent discrepancy between these two results is most likely due to the different membrane fractions analyzed. Elovson (10) isolated a "light" plasma membrane fraction in density gradients after vigorous mechanical disruption of what appeared to be a more sheetlike plasma membrane preparation (4). Therefore, the fraction analyzed probably consisted of vesicles derived largely from the apical domain (3). Others (10, 11, 44) have observed that <1 h of chase in vivo was required to achieve maximum specific radioactivities of protein-associated L-[<sup>3</sup>H]fucose, L-[<sup>35</sup>S]methionine, or L-[<sup>3</sup>H]leucine in their respective rat liver plasma membrane fractions.

### *Newly Synthesized Apical Proteins Are Present in Basolateral Plasma Membrane Vesicles at 45 min of Chase*

Our sucrose gradient and free-flow electrophoretic analyses of the domain localization of the newly synthesized plasma membrane proteins indicate that, at the time when the plasma membrane proteins first reach their maximum specific radioactivities in the plasma membrane fraction (at 45 min of chase), the radiolabeled, mature molecular mass forms of the apical proteins are not associated with the apical domain, but are present in different membrane vesicles.

These vesicles: (a) fractionate like basolateral plasma membrane using the two independent separation techniques employed (Figs. 2, 3, and 6); (b) can be separated from the bulk of the organellar contaminants that would be most likely to contain the mature molecular mass forms of the newly synthesized proteins, including late Golgi cisternae, the trans-tubular network, and endosomes (Table I and Figs. 5–7); (c) contain the proven basolateral constituents, the ASGP-R and CE 9 (Table II); and (d) are oriented with their ectoplasmic surfaces facing outward (Table II), characteristic of vesicles derived from the plasma membrane but not of those derived from intracellular organelles. Taken together, our data indicate that the vesicles containing the newly synthesized apical (and basolateral) proteins at 45 min of chase are bona fide basolateral plasma membrane.

Existing experimental evidence permits us to exclude the possibility that the radiolabeled apical proteins simply diffuse into the basolateral domain during the isolation of the plasma membrane sheets. In a previous study we used an immunogold labeling procedure to show that CE 9 and HA 4 retained their domain-specific distributions even on unfixed preparations of the isolated sheets (3). Furthermore, if there had been any such redistribution, then all of the proteins, radiolabeled or unlabeled, would have been expected to exhibit a bimodal distribution in the domain gradients. Although unlikely, given our evidence, we cannot presently rule out the formal possibility that we have significantly enriched vesicles for a population of intracellular transport that vesiculate with their ectoplasmic surfaces facing outward, coincidentally fractionate like basolateral plasma membrane in sucrose density gradients and in free-flow electrophoresis, and contain the ASGP-R. Kinetic experiments involving exogenous vectorial labeling of the newly synthesized apical proteins after their appearance at the basolateral surface followed by their detection in the apical domain would resolve this issue.

### ***Apical Proteins Move to the Apical Domain at Different Rates***

As judged by their distributions in the domain gradients (Figs. 2–4), significant amounts of the mature radiolabeled apical proteins, but not the radiolabeled basolateral proteins, begin to appear in the apical domain at chase times greater than 45 min. Interestingly, the newly synthesized apical proteins reach their apical destinations at different rates (Fig. 4). Approximate half-times of arrival are in the range of 90–120 min for AP and DPP IV, whereas HA 4 moves substantially slower than this, with 80–85% remaining basolateral even after 150 min of chase. It should be reiterated that all of the proteins maintain a nearly constant specific radioactivity in the plasma membrane fraction during this period of redistribution (Fig. 1). Recent studies of membrane biogenesis in cultured Caco-2 cells (derived from a human adenocarcinoma cell line [34]) suggest that disaccharidases and peptidases also appear at the apical surface at vastly different rates (reference 12; and Stieger, B., B. Baur, K. Bucher, M. Hochli, and H.-P. Hauri, manuscript submitted for publication).

### ***Mechanism of Sorting of Hepatocyte Plasma Membrane Proteins***

Our results suggest a mechanism for hepatocyte plasma membrane biogenesis in vivo in which every integral plasma

membrane protein, basolateral or apical, is shipped first to the basolateral domain, and then the apical proteins are delivered from the basolateral domain to the apical domain at different rates. At present, the resolution of apical and basolateral vesicles in the domain gradients and in free-flow electrophoresis is not sufficient to rule out a possible mechanism involving random protein appearance in both plasma membrane domains. However, the ratio of relative surface areas of basolateral and apical domains in the isolated hepatocyte plasma membrane sheets is approximately 7:3 (18), meaning that at least 70% of the newly synthesized apical proteins would arrive first at the basolateral domain and then need to be sorted to their proper final destination. One important implication of such a mechanism is that the sorting event(s) would need to occur after the initial insertion into the basolateral plasma membrane. Since the available evidence supports the notion that tight junctions restrict lateral protein diffusion between basolateral and apical domains (reviewed in reference 39), it is likely that the postulated basolateral-to-apical movement of hepatocyte plasma membrane proteins occurs by some form of vesicle-mediated transcytosis. Thus, the sorting of apical from basolateral proteins could be the result of selective endocytosis and transport. The actual sorting step might occur either with the selective endocytosis of newly synthesized apical proteins at the basolateral surface or at some intracellular site after the endocytosis of the newly synthesized apical proteins along with the basolateral constituents. Clearly, to explain our data, this vesicular transport between domains would need to be fast enough to account for the fact that there is no observable reduction in the specific radioactivity of these proteins in the plasma membrane sheet fraction during the relevant time period (Fig. 1). The transport would also have to occur at distinct rates for different apical proteins (Fig. 4). In this regard, the apical protein HA 4 presents us with a particularly intriguing case. A large percentage of the mature radiolabeled HA 4 (80–85%) remains basolateral after an extended period of chase (150 min), even though HA 4 is clearly localized apically in the steady state by both biochemical and morphological means (3, 18). These two observations are not incompatible given that preliminary estimates of HA 4's half-life (>30 h) are considerably longer than the chase times thus far examined (our unpublished data). However, the data do suggest that HA 4's basolateral-to-apical movement might either be more tightly regulated than those of the other apical proteins examined or that its movement might somehow be impaired by the experimental conditions, e.g., the fasting of the rats or the administration of relatively large quantities of unlabeled methionine. Further experimentation will be required to resolve this issue.

### ***Other Examples of Plasma Membrane Protein Sorting in Epithelial Cells***

There is precedent for the selective basolateral-to-apical movement of integral plasma membrane proteins in vivo in hepatocytes. One of the best examples is the receptor for polymeric immunoglobulin A, which during its normal life cycle binds circulating ligand at the basolateral surface, is endocytosed along with ligand and transported in vesicles to the apical pole of the cell, is inserted into the apical domain as these vesicles fuse with the apical plasma membrane, and then is proteolytically released into the apical lumen while

still complexed with polymeric immunoglobulin A (16, 31, 33, 40, 43). Transcytotic receptors for other ligands, such as the hemoglobin-haptoglobin complex (33), are believed to traverse a similar pathway. Based upon the observation that [<sup>3</sup>H]fucoproteins appeared in a sinusoidal plasma membrane subfraction before a canalicular subfraction, Evans et al. (11) were perhaps the first to postulate the existence of "a route involving direct transfer of glycoproteins via a membrane-mediated path from the blood-sinusoidal to the bile canalicular plasma membranes". However, this sinusoidal subfraction showed extensive contamination by Golgi, and mature forms of specific hepatocyte plasma membrane proteins were not considered.

Studies of plasma membrane biogenesis in the intestinal epithelial cell have yielded contradictory results regarding the pathway taken by apical proteins. Mokteri et al. (30) used a novel flow-cytometric technique to suggest a basolateral-to-apical pathway for newly synthesized AP in rabbit enterocytes, an observation reinforced by *in vivo* metabolic labeling (27). However, Danielsen and Cowell reported an opposite result for AP and several other brush border enzymes using immunoelectrophoretic separation of membranes (6). In addition, Lorenzsonn et al. (25) recently found no evidence for the presence of significant amounts of a different brush border enzyme, sucrase-isomaltase, at the basolateral surface of rat intestinal epithelial cells using immunocytochemical techniques that were sensitive enough to permit detection of the enzyme in the endoplasmic reticulum, the Golgi complex, and smooth vesicles in the apical cytoplasm.

Our findings would appear to be at odds with the dogma that has arisen from studies of RNA viral envelope glycoprotein sorting in cultured epithelial cell lines, such as Madin-Darby Canine Kidney (MDCK). It has been convincingly demonstrated in these cells, both morphologically and biochemically, that the newly synthesized envelope glycoproteins of apically budding viruses (e.g., HA of influenza) are inserted directly into the apical domain, with no detectable residence in the basolateral domain (for reviews, see references 28, 35, 39). Thus, in these cells, sorting of basolateral from apical proteins has been thought to occur in the late Golgi, after sialylation of the molecules (12). The results of our study clearly indicate that in rat hepatocytes sorting occurs at or after proteins reach the basolateral surface, which means that the biosynthetic pathways taken by apical molecules in the two cell types are different. Assuming that endogenous domain-specific proteins in MDCK cells behave like the viral molecules (as yet untested for an apical molecule), how can these results be reconciled? Examination of the secretory pathways for soluble molecules in the two cell types offers a clue.

Discrete sets of endogenous soluble proteins are constitutively secreted by MDCK cells into either the apical or basolateral media (5, 13, 23). Furthermore, a variety of exogenous secretory proteins synthesized in MDCK cells transfected with the corresponding genes are secreted into both the apical and basolateral media (13, 23). This even applies to the serum form of  $\alpha_{2u}$ -globulin, a liver protein that is normally secreted in a constitutive fashion from only the basolateral surface of rat hepatocytes. In these latter cells there is no evidence for the secretion of any newly synthesized proteins directly into bile, the apical environment of the

hepatocyte. The majority, if not all, of the proteins present in bile arrive there secondarily from residual lysosomes, the blood, or the sinusoidal (basal) surface (22, 24). Thus, in MDCK cells, two secretory pathways exist, while hepatocytes have only one.

The difference in the biosynthetic routes taken by secretory molecules parallels the difference in the biosynthetic pathways taken by integral plasma membrane proteins in these two cells. A number of interesting questions and predictions arise from this observation. (a) Are the constitutive routes of protein secretion and membrane protein expression the same in a particular epithelial cell? That is, do the same post-Golgi vesicles carry both newly synthesized secretory and membrane proteins to one surface domain? An immunoelectron microscopy study of nonpolarized hepatoma cells has colocalized representatives of these two protein classes in the same vesicles; however, the outward direction of the vesicles was not established (42). Kinetic experiments *in vivo*, together with subcellular fractionation, could answer this question for the hepatocyte. (b) How does the machinery for the sorting of membrane proteins destined for apical and basolateral domains differ in different epithelial cells? There has been considerable focus recently on the *trans*-Golgi as a sorting site and acidification as a mechanism for segregating molecules into different pathways (14, 21, 29). In the rat hepatocyte it would appear that at least the location and possibly the mechanism for sorting apical membrane proteins differ from those in epithelial cells where direct pathways to both surfaces exist. (c) Finally, are there different sorting signals on analogous apical plasma membrane proteins found in different epithelia? In this regard, it would be extremely interesting to compare the polypeptide sequences of such molecules as well as their biogenetic routes when introduced into the reciprocal cells (e.g., hepatocyte protein into MDCK cells). A likely candidate for such studies would be the apical protein AP, since it is present in several epithelia (e.g., see reference 26). It may turn out that cells that have no constitutive apical secretory pathway, such as the hepatocyte (22), will use the basolateral-to-apical transcytotic route for the delivery of all apical plasma membrane proteins to their apical domains.

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