Biogenesis of Plasma Membrane Glycoproteins

TRACER KINETIC STUDY OF TWO RAT LIVER PLASMA MEMBRANE GLYCOPROTEINS IN VIVO*

(Received for publication, March 28, 1979, and in revised form, October 30, 1979)

John Eloovson†

From the Department of Biology, University of California, San Diego, California 92083 and the Research Service, Veterans Administration Wadsworth Hospital Center, Los Angeles, California 90073

Antibodies to purified nucleotide pyrophosphatase (NPPase) and dipeptidyl peptidase IV (DPP IV) were used to study the biogenesis of these rat liver plasma membrane glycoproteins in vivo. Following injection of tritiated leucine, the radioactivity in NPPase and DPP IV decayed at markedly different rates in the plasma membrane, with apparent half-lives of about 1 and 5 days, respectively. In short term experiments, labeling of total plasma membrane proteins was rapid and insensitive to colchicine, while labeling of both NPPase and DPP IV showed a lag of about 15 min, followed by colchicine-sensitive/cycloheximide-insensitive increases to half-maximal and maximal values at about 1 and 2 h, respectively. A peak of labeled DPP IV in rough microsomes at 15 min showed increased mobility on polyacrylamide gels and was largely inaccessible to antibodies in intact microsomes, consistent with its being an underglycosylated precursor, exposed on the cisternal side of the rough endoplasmic reticulum. In contrast, the behavior of unlabeled DPP IV in preparations of rough microsomes and Golgi was consistent with its being contributed by contaminating right-side-out plasma membrane vesicles. This conclusion was also necessary to fit the tracer kinetic data to a simple membrane-flow model, which gave precursor pools (1 µg/g of liver) and fluxes (1 µg/h/g of liver) for both DPP IV and NPPase which were about 1 orders of magnitude less than those for the synthesis of rat serum albumin. Thus, unlike hepatoma tissue culture cells (Doyle, D., Baumann, H., England, B., Friedman, E., Hou, E., and Tweto, J. (1978) J. Biol. Chem. 253, 967-973), normal rat liver does not contain large amounts of preformed intracellular plasma membrane precursors.

According to the membrane-flow hypothesis (1), secretory proteins, which are made in the rough endoplasmic reticulum, are transported to the cell surface via the smooth endoplasmic reticulum and the Golgi apparatus, with a parallel flow of enclosing membranes. Many secretory proteins are glycoproteins and recent work from a number of laboratories has demonstrated how an integrated process of translation, translocation, and core-glycosylation delivers the nascent glycoproteins into the cisternal space of the rough endoplasmic reticulum (2-5); these nascent glycoproteins are then variously modified, notably by addition of terminalgalactose and sialic acid in the Golgi apparatus (6), before being externalized following fusion of Golgi secretory vesicles with the plasma membrane. It is now generally believed that the glycoproteins in the latter, which display their carbohydrate portions exclusively on the outer cell surface (7, 8), share the same membrane-flow pathway (7-10), differing from soluble glycoproteins in remaining associated with the equivalent of the extracellular/cisternal face of the membranes involved. However, there is little direct evidence on this point, particularly in regard to the actual kinetics of intracellular transport and turnover of individual plasma membrane glycoproteins in vivo. We have approached this problem by purifying two such proteins from rat liver and have used their antibodies to address three aspects of their biogenesis. 1) Do plasma membrane glycoproteins turn over at the same or different rates? 2) Is the kinetics of their biogenesis consistent with a membrane-flow mechanism? 3) Are plasma membrane glycoproteins also found as genuine components of intracellular membranes? Some of these results have been presented in abstract form (11).

EXPERIMENTAL PROCEDURES

The following procedures are described in the accompanying paper (12): preparation and assay of NPPase* and DPP IV and production of their antibodies in rabbits; detergent solubilization and immunoprecipitation of NPPase and DPP IV; quantitative and analytical SDS-polyacrylamide gel electrophoresis; and chromatography on Sepharose-coupled wheat germ agglutinin.

Treatment of Animals—Experiments were started at about 10:00 a.m. Ad libitum fed female Sprague-Dawley rats, about 200 g body weight, were lightly anesthetized with ether and injected intraperitoneally at 0.5 mg/kg body weight. When indicated, colchicine (1 mg/kg) was administered intraperitoneally at 2 h before the labeled leucine. In all short term experiments (4 h or less), cycloheximide (3 mg/kg body weight) was also given intraperitoneally 15 min after L-1-[3H]leucine. Animals were sacrificed by exsanguination under ether and their livers quickly perfused in situ with 37°C saline before removal at the indicated times.

Subcellular Fractions—Crude and light plasma membrane was prepared from rat liver as described (13). Samples of smooth microsomes and Golgi apparatus were prepared by the method of Leelavathi et al. (14) and rough microsomes as described by Giaumann et al. (15). The proportion of total liver protein assumed to be accounted for by the corresponding Golgi and endoplasmic reticular compartments in vivo was taken as the reciprocals of the enrichments over homogenate for appropriate marker enzymes (16): an average 50-fold for galactosyltransferase in Golgi (14, 15, 17) and 4.5-fold for cytochrome P-450 and NADPH-cytochrome c reductase in endoplasmic reticulum (15, 18, 19), with a 2:1 ratio of rough and smooth elements (20).

Determination of Specific Radioactivities—The distribution of

* The abbreviations used are: NPPase, nucleotide pyrophosphatase; DPP IV, dipeptidyl peptidase IV; SDS, sodium dodecyl sulfate; IgG, immunoglobulin G; HTC, hepatoma tissue culture cells.

† Address inquiries to V. A. Wadsworth Medical Center, Building 115/Room 316, Wilshire and Sawtelle Boulevards, Los Angeles, CA 90073.

‡ This investigation was supported in part by United States Public Health Service Grants AM 15836 and AM 21072. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
radioactivity in immunoprecipitates was determined by electrophoresis on 1 mm thick 7.5% to 12.5% polyacrylamide gradient slab gels (12 x 10 cm) using the Neville (21) discontinuous SDS buffer system. Depending on sample size, three to six precipitates were run per gel. After solubilizing samples as described (12), measured volumes containing 0.5 to 5 μg of antigen were applied to the slab gel and electrophoresed at 2.5 mA until the tracking dye entered the lower gel, at which point the current was increased to 7.5 mA. The gels were fixed and stained by slow agitation in 0.025% Coomassie blue R in 2% isopropanol alcohol, 10% acetic acid for 1 h, followed by overnight staining in 10% acetic acid. Sample lanes were cut out and an array of regularly spaced razor blades were used to divide areas of interest into approximately 1.2-mm slices. Each slice was chopped into 1-mm pieces, transferred to a scintillation vial containing 1 ml of ICN tissue solubilizer, and the capped vial heated at 80°C for 5 h. After cooling to room temperature, 10 ml of Econofluor were added and the samples stored overnight in the refrigerated scintillation counter before counting to less than 10% error. Under these conditions, the gel pieces swell several fold in size, with quantitative release of labeled proteins into the scintillation mixture. The specific activity (disintegrations per min per mg) of immunoprecipitated NPPase and DPP IV was calculated as the total radioactivity associated with the respective peptide divided by its mass, as determined by quantitative preparative slab gel and analytical cylindrical gel electrophoresis, respectively. All radioactive measurements have been normalized to a standard injection of 1 mCi/200 g body weight.

To determine total protein radioactivity samples were precipitated and washed three times with 10% (w/v) trichloroacetic acid, followed by ethanol/diethyl ether (1:4) and ether. The pellets were solubilized by heating in 1 ml of ICN tissue solubilizer and counted as described above.

Disposition of DPP IV in Subcellular Fractions—The accessibility of labeled and unlabeled DPP IV to its antibody before and after detergent treatment was estimated as follows. Preparations of rough microsomes, Golgi, and light plasma membrane in phosphate-buffered saline were treated with excess anti-DPP IV IgG at room temperature for 20 min, after which unbound IgG was removed by three cycles of centrifugation (Sorvall SS-34, 20,000 rpm, 30 min) and resuspension in the same medium. The washed suspensions, 1 mg of protein/ml, were then chilled on ice, lysed by addition of ½ volume each of 20% (w/v) TritonX-100 and 5 M NaCl, and immediately centrifuged as above to give the first immunoprecipitates, which contained DPP IV which was available to the antibody before detergent lysis. Addition of a second portion of antibody to the supernatants gave a smaller second immunoprecipitate, which contained antigen which had been inaccessible to the antibody in the original unlysed particles.

Other Methods—Protein was determined by the Lowry method as described (13). Desialylated DPP IV was prepared by treatment of the wheat germ agglutinin-adsorbable fraction (12) of a crude plasma membrane lysate with protease-free neuraminidase, essentially as described by Winkelbrak and Nicoolson (22).

Kinetic Model—A linear model for the synthesis and turnover of plasma membrane glycoproteins is shown in Fig. 6. The movement of a labeled protein component, such as DPP IV or NPPase, after its synthesis from labeled free leucine (Leu), through the compartments, is given by Equations 1 to 4:

\[
\frac{dr}{dt} = \left( F_{lw}(t) - a \cdot \frac{R}{R} \right) \cdot j
\]

\[
\frac{ds}{dt} = \left( \frac{R - s}{R - S} \right) \cdot j
\]

\[
\frac{dg}{dt} = \left( \frac{s - g}{S - G} \right) \cdot j
\]

\[
\frac{dp}{dt} = \left( \frac{g - p}{G - P} \right) \cdot j
\]

where \( j \) is the flux of the component (milligrams of protein/h/g of liver), \( F_{lw}(t) \) is the specific radioactivity (disintegrations per min/μmol) of free leucine in the liver at time \( t \), \( a \) is the amount of leucine in the component (micromoles of leucine/μg of protein), and where \( R, S, G, \) and \( P \) represent the mass (milligrams of protein/g of liver) and \( r, s, g, \) and \( p \) the radioactivity (disintegrations per minute/g of liver) of the component present in the rough endoplasmic reticulum, smooth endoplasmic reticulum, Golgi, and plasma membrane compartments, respectively. Equations 1 to 4 were solved numerically using a digital computer. The function \( F_{lw}(t) \) was constructed by linear interpolation between the data provided by Peters and coworkers (23, 24) and Poole (25) normalized to a standard injection of 1 mCi of [3H]leucine/200 g rat; for reasons given under "Results," Poole's values were also uniformly reduced by one-third. When cycloheximide was given at 15 min after the labeled leucine, \( F_{lw}(t) \) was set to zero from 16 min. The value of \( a \) for DPP IV was from the accompanying paper (12) and was also arbitrarily applied to NPPase, for which no amino acid analysis was available.

L-[4,5-3H]Leucine (50 Ci/mmol), tissue solubilizer, Econofluor, and Aquasol were obtained from ICN. Colchicine, cycloheximide, and Clostridium perfringens neuraminidase, type V, were from Sigma Chemical Co.

RESULTS

Apparent Rate of Turnover of Liver Plasma Membrane Proteins—After injection of labeled leucine, the total proteins of crude liver plasma membrane decayed over several days at progressively slower rates, as expected for a mixture of components with different turnover (Fig. 1), with the first and last experimental intervals corresponding to apparent half-lives of about 1.2 and 6 days, respectively, without consideration of label reutilization. For the individual components, the time course in DPP IV and the early part of that in NPPase could be reasonably fitted by eye to single exponential decays, corresponding to uncorrected half-lives of about 5 and 0.9 days, respectively. Thus, these two externally disposed2 glycoproteins fell in the categories of most slowly and rapidly turning over plasma membrane proteins, respectively. Corrections to these half-lives for label reutilization are discussed below.

Early Time Course for Labeling of Plasma Membrane Proteins—Fig. 2 shows the initial incorporation of radioactivity into light plasma membrane components after injection of tritiated leucine. It is seen that a 15-min lag occurred before significant radioactivity was detected in NPPase and DPP IV. Although protein synthesis was arrested at that point by injection of cycloheximide, the specific radioactivity in these
two glycoproteins then proceeded to increase roughly in parallel and to level off toward 2 h, although in keeping with their widely different half-lives, the specific activity of DPP IV was about one-tenth of that for NPPase throughout this period. In contrast, the unfraccionated plasma membrane proteins showed a rapid initial rise in specific activity, reaching half-maximal values before 15 min and leveling off between 1 and 2 h. This rapid rise could not be accounted for by the estimated 10% contamination with microsomes (13) since the specific activity of their membrane proteins at 15 min was at most twice that of the light plasma membrane (data not shown).

Since the time course in Fig. 2 was reminiscent of that for the secretion of plasma proteins from the liver, which is inhibited by colchicine (26), the effect of this compound on the translocation of newly synthesized NPPase and DPP IV to the plasma membrane was also tested (Fig. 3). This experiment was complicated by the fact that the total acid-insoluble activity in liver plus plasma of colchicine-treated animals was only about one-half of that in the untreated controls; a similar effect of colchicine on liver protein synthesis was recently reported by Redman et al. (27). To compensate for this effect, the protein specific radioactivities in treated animals at each time point were, therefore, multiplied by the factor required to make this total radioactivity equal to that in the corresponding control animals. It is seen in Fig. 3 that even with such correction colchicine markedly inhibited not only the secretion of radioactive plasma protein but also, in roughly parallel manner, the rate of appearance of labeled NPPase and DPP IV in the plasma membrane; the rate of appearance of total protein radioactivity in the latter, on the other hand, was not significantly affected by the drug.

Subcellular Distribution of NPPase and DPP IV—Since the membrane-flow hypothesis postulates rough and smooth endoplasmic reticulum and the Golgi apparatus as precursor compartments for the plasma membrane, the concentration of NPPase and DPP IV in the corresponding subcellular fractions was also determined (Table I). It is seen that the enrichment relative to homogenate is quite similar for the two proteins both in crude and light plasma membrane preparations and in good agreement with our previous values for NPPase (13). Their abundance in preparations of rough and smooth microsomes, taken as representative of rough and smooth endoplasmic reticulum, as well as in Golgi preparations, was much less than in the plasma membrane (Table I), raising the question whether this merely represented cross-contamination of the former with the latter or whether such surface proteins also were present to this extent as genuine components of the intracellular membranes. Thus, it is seen from Table I that if the latter were the case, the endoplasmic reticulum, rather than the plasma membrane, would account for the better part of the liver's content of DPP IV and NPPase. In this regard, it is shown in the following sections that the bulk of these proteins in the intracellular fractions behaved as expected for contaminating plasma membrane elements and could not be intermediates in de novo synthesis.

**Appearance and Properties of Labeled, Immunoprecipitable DPP IV in Subcellular Compartments**—Fig. 4 shows the normalized specific radioactivity of DPP IV recovered by immunoprecipitation from detergent lysates of rough and smooth microsomes, Golgi, and plasma membrane at various times up to 2 h after injection of [H]leucine; unfortunately, the supernatants from these experiments, originally saved for immunoprecipitation of NPPase, were mistakenly discarded. The specific activity of DPP IV in rough microsomes peaked rapidly at 15 min, a time when no label had yet reached the plasma membrane; the label in the former then decayed while that in the latter increased so that specific activities in the two compartments approached the same value at 2 h. That in the Golgi preparations showed an intermediate behavior, rising earlier than in the plasma membrane, to level off at values somewhat higher than those for the latter at later times. At

---

**Fig. 2. Appearance of newly synthesized DPP IV, NPPase, and total protein in rat liver light plasma membrane after injection of L-[^3H]leucine.** Each symbol represents a sample from pooled livers of two to three animals and was obtained in three separate experiments, one of which was the control experiment shown in Fig. 3. The specific radioactivities of total serum protein, NPPase, and DPP IV are shown. Arrow shows injection of cycloheximide. Note different ordinate scales.

**Fig. 3. Effect of colchicine on intracellular transport of newly synthesized serum and plasma membrane proteins in rat liver.** Colchicine, 1 mg/kg body weight, or the equivalent volume of saline was injected intraperitoneally at 3 h before injection of L-[^3H]leucine. Ordinates show the specific radioactivities of total serum proteins (a), total plasma membrane proteins (b), plasma membrane NPPase (c), and plasma membrane DPP IV (d). Note different ordinate scales. Symbols in a represent samples from individual animals in b to d those from pooled livers of two animals. Arrow as in Fig. 2.
15 min, the specific activity of DPP IV in smooth microsomes was higher than in Golgi and then continued to rise slowly through 60 min, the latest point sampled.

The radioactive DPP IV which peaked in rough microsomes at about 15 min differed in two respects from the bulk of the unlabeled DPP IV in the same samples. Fig. 5 shows the distribution of radioactivity and mass in the pertinent area of the preparative SDS-polyacrylamide slab gel of immunoprecipitated DPP IV from lysates of rough microsomes at 15 and 120 min after [3H]leucine injection. Within the resolution of the system, the peaks of radioactivity and protein overlapped completely for the later sample, as was also the case for samples from Golgi and plasma membrane lysates (not shown); however, in the rough microsome sample, at 15 min, the specific activity of DPP IV in smooth microsomes was higher than in Golgi and then continued to rise slowly through 60 min, the latest point sampled.

Table I

<table>
<thead>
<tr>
<th>Protein</th>
<th>mg/g liver</th>
<th>DPP IV</th>
<th>mg/g protein</th>
<th>NPPase</th>
<th>mg/g liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate Crude plasma membrane</td>
<td>175 ± 12</td>
<td>49 ± 2 (6)</td>
<td>10 ± 1.2 (15)</td>
<td>66</td>
<td>0.08 ± 0.03 (7)</td>
</tr>
<tr>
<td>Light plasma membrane</td>
<td>37 ± 6 (6)</td>
<td>1.2 ± 0.3 (6)</td>
<td>32</td>
<td>0.22 ± 0.03 (5)</td>
<td>6</td>
</tr>
<tr>
<td>Rough endoplasmic reticulum</td>
<td>26</td>
<td>1.9 ± 0.4 (3)</td>
<td>25</td>
<td>0.23 ± 0.04 (3)</td>
<td>3</td>
</tr>
<tr>
<td>Smooth endoplasmic reticulum</td>
<td>3.5</td>
<td>2.3 ± 1.0 (11)</td>
<td>8</td>
<td>0.83 ± 0.33 (11)</td>
<td>3</td>
</tr>
<tr>
<td>Golgi</td>
<td>3.5</td>
<td>2.3 ± 1.0 (11)</td>
<td>8</td>
<td>0.83 ± 0.33 (11)</td>
<td>3</td>
</tr>
</tbody>
</table>

* Calculated from enzymatic activity, assuming 1200 units/mg for the pure protein (15).
* Mean ± S. D. (number of samples).
* Rough microsomes (15).
* Calculated as described under “Experimental Procedures.”
* Smooth membranes (14).

The appearance of newly synthesized DPP IV in different liver subcellular fractions after injection of [3H]leucine. Ordinate shows specific activity of DPP IV in rough endoplasmic reticulum (●), smooth endoplasmic reticulum (▲), and Golgi (○). Dashed line is DPP IV specific activity in plasma membrane, redrawn from Fig. 2. Each symbol represents a sample from pooled livers of two to three animals. Arrow as in Fig. 2.

The radioactivity migrated slightly but significantly faster than the stained peptide. As seen in the inset of Fig. 5, removal of sialic acid from DPP IV also produced a small but definite increase in its electrophoretic mobility, suggesting that the radioactive species found in rough microsomes at 15 min could lack sialic acid and perhaps other terminal sugars, as expected for a precursor in the rough endoplasmic reticulum, which would have the asparagine-linked core saccharides but lack galactose and sialic acid (4, 6). A similar phenomenon has been shown for the nascent glycoprotein of the vesicular stomatitis virus envelope (9, 28). The large amount of unlabeled DPP IV found in the rough microsome sample at 15 min, as well as at other times, could, therefore, not be a biosynthetic intermediate at that level since the stained DPP IV peptide in the immunoprecipitates from all subcellular fractions had a mobility indistinguishable from that of the mature sialoprotein found in the plasma membrane; the presence of this material in rough microsomes would, therefore, most simply be accounted for as contamination with plasma membrane fragments, particularly since the DPP IV specific activity in the two fractions eventually became the same.

The disposition of labeled DPP IV in rough microsomes at early times was also different from that of the bulk DPP IV mass. We have shown elsewhere that all determinants recognized by the anti-DPP IV used here were located on the extracellular surface of the liver plasma membrane, corresponding to the inner cisternal surface of endoplasmic reticulum and Golgi. An attempt was, therefore, made to demonstrate such crypticity by treating subcellular fractions with the antibody before and after detergent lysis, as described under “Experimental Procedures.” The first immunoprecipitates obtained corresponded to DPP IV directly available to antibody in the intact fraction, the second to DPP IV which became available only after lysis. The proportion of immunoprecipitable mass which was recovered in the first precipitate and the ratios of specific radioactivities of the two immunoprecipitates are shown in Table II as measures of the
relative crypticity of newly synthesized (labeled) and pre-
formed (unlabeled) DPP IV. The data will not be taken as
absolute measures of the inside-outside disposition of this
antigen since samples had been stored in liquid nitrogen,
which may have caused some loss of membrane integrity;
however, it is seen that the specific activity ratio was close to
unity in plasma membrane and that almost all of its DPP IV
was accessible to antibody before detergent treatment, con-
sistent with our previous finding that this preparation carried
DPP IV exclusively on right-side-out vesicles. The same was
true for a Golgi preparation sampled at a late time, most
simply accounted for by assuming that it contained DPP IV
largely as contaminating vesicles. On the other hand, although
the bulk of the nonradioactive antigen in rough microsomes
also was accessible to antibody without prior lysis, the tran-
sient peak of labeled DPP IV at early times was largely cryptic
and was recovered mostly in the second precipitate obtained
after detergent treatment. At 120 min this difference had
essentially disappeared, again consistent with the suggestion
that at that point the presence of both labeled and unlabeled
DPP IV in rough microsomes was predominantly due to
contamination with right-side-out plasma membrane vesicles.

**Kinetic Treatment**—The linear model shown in Fig. 6 is the
simplest statement of the membrane-flow hypothesis for
plasma membrane biogenesis. As applied to glycoproteins, it
postulates translation in rough endoplasmic reticulum, se-
quential passage through smooth reticulum and the Golgi
apparatus with appropriate further glycosylation, and final
insertion into the plasma membrane by fusion of Golgi-derived
vesicles with the latter. It is assumed that such proteins
maintain a strict orientation throughout this pathway, with
their glycosylated portions exposed on the extracellular (in-
tracellular) face of the respective membranes. The model
considers only a unidirectional linear flow of newly made
protein with a single flux \((j)\) through all compartments, equal
to the irreversible loss from the plasma membrane; additional
branching and exchanging pathways will be considered under
"Discussion." In the simplest analysis, these fluxes for DPP
IV and NPPase may, therefore, be calculated directly from
the size and estimated true half-lives of their plasma mem-
brane pools. Regarding the latter, with guanidino-
labeled arginine suggests that use of leucine overestimated
half-lives for liver plasma membrane proteins by an average
factor of about 2 (29), although this correction itself also will
vary with the half-life of the protein in question (25). With
this correction to the apparent half-lives, which were obtained

### Table II

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Time after 1 Hleucine (min)</th>
<th>DPP IV G. of total mass in first precipitate</th>
<th>Specific activity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma membrane</td>
<td>120</td>
<td>85</td>
<td>0.86</td>
</tr>
<tr>
<td>Golgi</td>
<td>120</td>
<td>87</td>
<td>0.80</td>
</tr>
<tr>
<td>Rough microsomes</td>
<td>7</td>
<td>68</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>93</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>87</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>82</td>
<td>0.74</td>
</tr>
</tbody>
</table>

**Fig. 6.** Linear membrane-flow model for plasma membrane
biogenesis. The flux \((j)\) of a newly synthesized protein component
is the same through each compartment and equal to the rate of
irreversible removal from the plasma membrane.

Graphically in Fig. 1, and with the assumption that essentially
all of the DPP IV and NPPase found in the liver (Table I)
properly belongs to the plasma membrane, the maximum
fluxes for both of these proteins may be calculated to be about
1 μg each/h/g of liver (Table II, Part A). In the simplest
analysis, the precursors of DPP IV or NPPase in the intracel-
lar compartments may then be lumped and treated as single
pools, with half-lives of approximately 1 h as defined by the
time required for both proteins to reach half-maximal specific
activities in the plasma membrane (Fig. 2). As shown in Table
II, Part A, such fluxes and half-lives correspond to the pres-
ence of about 1.3 μg of precursor each for DPP IV and
NPPase/g of liver, which is roughly 1 order of magnitude less
than the total amounts calculated to be present in our pre-
parations of intracellular membranes (Table I). As stated
above, it may, therefore, be concluded on kinetic grounds that
the bulk of DPP IV and NPPase which is found in such
preparations cannot represent intermediates in the transport of
the newly synthesized proteins to the plasma membrane.

To test their internal consistency, the data in Figs. 1, 2, and
4 were also fitted to the model in Fig. 6, as defined by
Equations 1 to 4 under "Experimental Procedures." For this
purpose, the specific activities were first converted into total
activities per gram of liver, with appropriate corrections for
the assumption that measurable NPPase and DPP IV mass
in all fractions actually represented plasma membrane ma-
terial. Values for the specific activity of free leucine in liver up
to 2 h after injection of a normalized dose of 1 mCi/200 g body
weight were taken directly from Peters (23, 24). The norma-
ized data given by Poole (25) for the remaining period up to
8 days did not show the required (30) intercept with the
maximum of the specific activity curve for leucine in DPP IV;
as discussed below, these values were, therefore, reduced by one-third, the minimum adjustment required for an acceptable intercept.

The fit for different fluxes to the decay of total activities in plasma membrane DPP IV and NPPase is shown in Fig. 7. It is seen that both the slope and ordinate position of the curve for DPP IV fit a flux of 0.8 µg/h/g of liver, corresponding to a half-life of 3.2 days, values very similar to those obtained above with the adjustment of the apparent half-life by one-half (Table III, Part A). As expected for a protein with a very short apparent half-life (25), the computed decay curves for NPPase were much more closely spaced (Fig. 7b), becoming indistinguishable at higher fluxes due to rapid equilibration of leucine in NPPase with the free leucine pool. As discussed by Poole (25), better estimates of its half-life require sampling at earlier times; as they stand, the data indicate a flux of 1 to 2 µg/h/g of liver, again similar to the value obtained above (Table III, Part A) by assuming the true half-life to be one-half of the apparent one.

The short term labeling of DPP IV and NPPase was next modeled. It is seen in Fig. 8, a and b that the flux determined in Fig. 7 gave a good fit for DPP IV in both plasma membrane and rough endoplasmic reticulum if the total size of its precursor compartments was taken as 0.8 µg/g of liver, distributed in a ratio 0.5:0.2:0.1 between rough and smooth endoplasmic reticulum and Golgi, so as also to fit the few data points for the smooth reticulum and the early values in Golgi; it is also seen that increasing or decreasing these values by a factor of 2 gave less satisfactory fit in rough endoplasmic reticulum and plasma membrane. For clarity, the similar changes in smooth reticulum and Golgi and the curves for different DPP IV fluxes are not included in Fig. 8, but it is obvious from Equations 1 to 4 and the rapid decay of radioactivity in free endoplasmic reticulum curve in a was obtained when the precursor pool of DPP IV in that compartment was set equal to the 32 µg/g of liver actually found in preparations of rough microsomes (Table I); the corresponding curve for plasma membrane DPP IV in b coincided with the abscissa. The time course in c for total NPPase radioactivity in the plasma membrane was computed for a single flux of 1.5 µg/h/g of liver (Fig. 7b), with the indicated total amounts (micrograms/g of liver) of NPPase precursor in rough and smooth endoplasmic reticulum and Golgi. Data points are total activities, calculated from average specific activities in Figs. 2 and 4 as described under "Results," with corrections for plasma membrane contamination of rough endoplasmic reticulum, smooth endoplasmic reticulum, and Golgi.
leucine (24) that both the total amount of radioactivity passing through the model, as well as the peak values in rough endoplasmic reticulum at early times and the plateau values in plasma membrane at late times, will vary almost linearly with the flux. Thus, only combinations of flux and pool size which are fairly close to those given above (Table III, Part B) will generate both the appropriate time course and peak values for rough reticulum and plasma membrane. The fact that this flux value was obtained from Fig. 7, therefore, shows that the model in Fig. 6 can account for both the short and long term kinetics of DPP IV in these major compartments. The pool size assigned to the smooth endoplasmic reticulum is provisional, considering the limited number of data points and the large correction for plasma membrane contamination; it would also be desirable to test the crypticity of the labeled DPP IV in this compartment. It is also clear that the simple model in Fig. 6 cannot give a good fit both to the early and late data points in Golgi, but at this stage, rather than trying to improve the fit with several Golgi compartments, this matter is best left open for more direct study in the future.

Since no data were obtained in the short term experiment on the availability of radioactive NPPase in rough and smooth microsomes or Golgi, the best distribution of the total intermediate pool of NPPase between these compartments could not be modeled. However, assuming it to be the same as that for DPP IV, and taking 1.5 µg/g of liver as an estimate of the NPPase flux in Fig. 7b, a total precursor pool of about 2 µg/g liver would fit the plasma membrane data in the short term experiments (Fig. 8c). Thus, according to the model, labeled NPPase and DPP IV appeared in the plasma membrane in parallel (Fig. 2) because both the flux and precursor pool of the former were about twice as large as those of the latter.

Regarding the validity of our using normalized literature data for the specific activity in free leucine, such values were reported to be quite reproducible for the 1st h after intravenous injection of the label and to be little affected by changes in the nutritional state of the animal (24). On the other hand, the long term values taken from Poole (29) by definition could not be directly fitted to our model since they were too high to intersect the specific activity curve for leucine in DPP IV when the latter was at its maximum, as required for their precursor-product relationship (30), nor was it possible to fit this data to the total DPP IV activities in Fig. 7a for any one DPP IV flux. We, therefore, reduced Poole’s values by one-third, which gave both an acceptable specific activity intercept (at 0.8 day) and a unique DPP IV flux, while further reduction, to one-half or less, again made it impossible to fit any one flux to the data in Fig. 7a. Thus, our model defined the required one-third reduction quite narrowly and as one which reasonably might be attributed to normal variations in leucine specific activities between independent experiments.

DISCUSSION

Much useful inference has been drawn from work on protein secretion and the assembly of some enveloped viruses (1-5, 9, 28), but direct studies on the synthesis and degradation of individual plasma membrane proteins have been few. The preparation of pure plasma membrane fractions in reasonable yield remains problematic in many systems, and the difficulties in purifying individual amphipathic integral components from this scarce material are obvious. Much work has been done on tissue culture cells, taking advantage of the unique accessibility of the outer cell surface to covalent labeling and enzymatic modifications. However, perhaps due to differences in cell lines and growth conditions, it is not clear to what extent the plasma membrane proteins in such cells turn over at the same or different rates or indeed at all (31-36), and the relevance of such findings to the very different conditions of most cells in vivo may itself be questioned (see below). The scarcity of material has also made it difficult to study the kinetic behavior of individual plasma membrane proteins in culture with the notable exception of fibronectin (37, 38) which, however, is largely associated with the extracellular matrix, rather than with the plasma membrane proper (39, 40).

In view of the above, rat liver was chosen for study here since its plasma membrane could be prepared in sufficient amounts to attempt the isolation of individual protein components. Even so, the low abundance of DPP IV, and particularly of NPPase, created difficulties both in the original isolation of the pure protein and in the tracer kinetic experiments themselves due to the correspondingly low incorporation of labeled leucine in vivo. Thus, the maximum rates of synthesis for either protein, in the order of 1 µg/h/g of liver, were about 3 and 4 orders of magnitude lower than those reported by Peters (23) for rat serum albumin and total liver protein, respectively. With the added difficulties encountered in conventional purification of these amphipathic proteins, the high yield and resolution of immunoprecipitation compared with SDS-polyacrylamide gel electrophoresis were indisputable for tracer kinetic studies. Although the antibodies to NPPase and DPP IV were highly specific, immunoprecipitation alone was not adequate, since the traces of contaminants detected in the precipitates by protein staining were accompanied by a disproportionately high background of radioactivity in parts of the gel other than the areas corresponding to the antigens, which was unaffected by previous removal of a “nonspecific” immunoprecipitate (rat serum/rabbit anti-rat serum). As might be expected, both background staining and labeling were more intense when double immunoprecipitation with goat anti-rabbit IgG was used, so much so that samples with low levels of label in DPP IV and NPPase could not be satisfactorily evaluated; results presented here are, therefore, only those obtained by single immunoprecipitation.

Three major conclusions may be drawn from the results presented here. One is that the two liver plasma membrane glycoproteins DPP IV and NPPase have widely different half-lives, as do populations of the unFractionated total proteins. This finding, which is obvious from Fig. 1 and not substantially affected by the corrections chosen for leucine reutilization in Table III, may be compared to the results obtained with the double isotope ratio method introduced by Arias et al. (29). Dehlinger and Schimke (41) found a positive correlation between apparent polypeptide size on SDS-polyacrylamide gels and their rate of turnover in rat liver plasma membrane, although it was seen to a lesser degree than in the microsomal and soluble fractions. Evans and Gurdon (42) claimed that a mouse liver plasma membrane fraction enriched in glycoproteins had a higher turnover rate than the total protein, and that the former also showed positive correlation between size, as determined by gel filtration in SDS, and turnover rate. However, the isotope ratios in these fractions differed only by about 10% and were so similar to those seen in control animals, which received both isotopes simultaneously, as to suggest that no significant turnover had occurred in either fraction. On the other hand, using SDS gel electrophoresis of liver plasma membrane, Landry and Marceau (43) found large and similar differences in isotope ratios both with leucine and glucosamine or fucose as precursors, which, however, did not correlate with the size of the peptides. While it is difficult to provide a single interpretation of these results, it is obvious that when the sample is a complex mixture, such as unFractionated plasma membrane, the limited resolution of slicing
Individual rat liver plasma membrane glycoproteins, such as DPP IV and NPPase, turn over independently, rather than concomitantly with the liver cells. The most straightforward interpretation of the liver permeability flux data, and certainly does not support their mutually exclusive localization to different domains, such as the blood or bile fronts. The most likely explanation of these data is that both proteins are found in isolated hepatocytes in liver, and from specialized larger domains in the same cell, such as the sinusoidal and bile canalicular surfaces in the hepatocyte. However, of these two hypotheses, the one that is the most likely situation in other cells as well, including rat liver in vivo (36). In their view, apparent heterogeneous turnover in such systems could plausibly be accounted for either by the plasma membrane preparations’ being contaminated with endomembranes or by their being a mixture, derived either from different cells in the same tissue (such as hepatocytes and Kupffer cells in liver) or from specialized larger domains in the same cell (such as the sinusoidal and bile canalicular surfaces in the hepatocyte). However, of these explanations, the first two clearly cannot account for the widely different half-lives found for DPP IV and NPPase since both proteins are found in isolated hepatocytes in amounts similar to those in liver (12) and have been shown to be located on the outer hepatocyte surface by immunofluorescence staining. We have also used anti-DPP IV–mediated density perturbation to show that most of the NPPase in our light plasma membrane preparation is located in vesicles which also contain DPP IV; although some redistribution of components during the preparation of this fraction could not be excluded, this argues against a markedly different distribution of these two proteins in the original plasma membrane and certainly does not support their mutually exclusive localization to different domains, such as the blood or bile fronts of the liver cells. The most straightforward interpretation of our findings, therefore, remains one which assumes that individual rat liver plasma membrane glycoproteins, such as DPP IV and NPPase, turn over independently, rather than concomitantly as suggested by Tweto and Doyle (36). It may be mentioned that the only other reported half-life for such a protein is 20 days for nicotinamide dinucleotide hydrolase (44), although this may require re-examination since the protein isolated was heterogeneous.

A second conclusion concerns the role of membrane-flow mechanisms in liver plasma membrane biogenesis. The precursor-product relationships entailed in such mechanisms have two aspects: that newly synthesized components first are found in other membranes of the cell, and the corollary that a lag, therefore, occurs before such components appear in the plasma membrane itself. Previous kinetic studies could only address the second aspect since they dealt with plasma membrane preparations, rather than with individually recognizable plasma membrane proteins, and, therefore, a priori had no means of identifying such proteins in other subcellular fractions. For liver, evidence of this limited kind was provided by the early work of Lieberman and co-workers (45, 46) and Franke et al. (47). Both groups found a lag before labeled amino acids appeared in the plasma membrane, which suggested that a membrane-flow mechanism was responsible for the biogenesis of most of its proteins. In contrast, we observed rapid labeling of the total plasma membrane protein, and it was also recently shown that while the incorporation of labeled glucose into HeLa cell plasma membrane showed a lag of about 12 min, this lag was only about 2 min for labeled leucine (48). We, therefore, propose, in contrast to the above conclusions (45–46), that a major part of newly made radioactive proteins in unfractionated liver plasma membrane arrives not via a membrane-flow pathway, but rather represents internally disposed peripheral proteins such as the large actin component, which could diffuse directly through the cytoplasm from their point of synthesis to their respective binding sites on the inner plasma membrane surface. This would also be consistent with our finding that the rate of labeling of total plasma membrane protein was not significantly affected by colchicine treatment, which did inhibit labeling of plasma membrane DPP IV and NPPase in parallel with that of the secretion of labeled proteins into serum. On this view the results of Lieberman and Franke and co-workers (45–47) could perhaps be accounted for if their methods of plasma membrane preparation removed the greater portion of such peripheral proteins, leaving predominantly amphipathic proteins as exemplified by DPP IV and NPPase. Again, this point could best be resolved by kinetic tracer analysis of individual peripheral proteins.

In contrast, the biogenesis of the integral glycoprotein DPP IV, and to the extent analyzed NPPase also, is found to fit a simple membrane-flow model. Here, the purification of these proteins and the preparation of their antibodies were prerequisites for determining the intracellular loci of glycoprotein precursors. This analysis was only completed for DPP IV, but the result clearly showed that the rough endoplasmic reticulum was the first and major precursor compartment. To the best of our knowledge, this is the first demonstration of this fact for a normal plasma membrane glycoprotein in an animal cell and does not support the proposal that such proteins could be translated and inserted into membranes at the level of the Golgi apparatus (49). Thus, the disposition, electrophoretic mobility, and kinetic behavior of the DPP IV precursor in rough endoplasmic reticulum were consistent with its being translated, vectorially inserted, and core-glycosylated in a manner similar to that recently detailed for the vesicular stomatitis virus envelope glycoprotein in vivo and in vitro (9, 28). As already discussed, the passage of newly made DPP IV through the smooth reticulum and Golgi was less well defined by the available data; however, although the well established subdivision of the Golgi apparatus a priori suggests a more complex model than that in Fig. 6, the data indicate that in any model the total amount of DPP IV intermediate in the

### Table III

<table>
<thead>
<tr>
<th></th>
<th>DPP IV</th>
<th>NPPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma membrane pool size, µg/g liver*</td>
<td>0.086</td>
<td>0.015</td>
</tr>
<tr>
<td>A. Estimated by graphic analysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t½ in plasma membrane, apparent † (days)</td>
<td>5</td>
<td>0.9</td>
</tr>
<tr>
<td>t½ in plasma membrane, estimated † (days)</td>
<td>2.5</td>
<td>0.45</td>
</tr>
<tr>
<td>Flux, µg/h/g liver †</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total precursor pools (rough and smooth endoplasmic reticulum and Golgi, µg/g liver †</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>B. Computed from model †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t½ in plasma membrane (days)</td>
<td>3.1</td>
<td>0.29</td>
</tr>
<tr>
<td>Flux (J), µg/h/g liver</td>
<td>0.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Precursor pools (µg/g liver)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rough endoplasmic reticulum</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Smooth endoplasmic reticulum</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Golgi</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.8</td>
<td>1.8</td>
</tr>
</tbody>
</table>

* From Table 1, assumed equal to total liver pools.
† From Fig. 1.
‡ t½ × apparent t½.
* Pool size × 0.693/t½ × 1.24.
$ Flux/0.693$ × estimated t½ of lumped pool. See text.
§ Fig. 6, Equations 1 to 4 under "Experimental Procedures."
Golgi apparatus must be small compared to that found in the rough endoplasmic reticulum.

A third conclusion concerns the possible presence of plasma membrane components such as DPP IV and NPPase in other subcellular fractions. As already discussed, the kinetic analysis clearly showed that the bulk of the DPP IV and NPPase which was found in rough and smooth microsomes or Golgi could not be precursors in the de novo synthesis of their plasma membrane counterparts; furthermore, direct analysis of rough microsomes showed that the labeled precursor form of DPP IV had a different electrophoretic mobility and disposition than did the mature unlabeled protein. It was, therefore, concluded that the large amount of mature DPP IV and NPPase found in the preparations of intracellular membranes most likely represented contamination with discrete plasma membrane elements, although the kinetic analysis alone could not exclude other interpretations. The simplest of such alternative formulations would allow large amounts of mature DPP IV and NPPase as genuine components of intracellular native formulations would allow large amounts of mature DPP IV and NPPase found in smooth microsomes or Golgi, which was found in rough and smooth microsomes or Golgi did not require them to rapidly mix with their plasma membrane counterparts, which was seen not to be the case when the latter were labeled by iodination of the intact cell. At this point, therefore, there appears to be a genuine difference between the biogenesis of plasma membrane glycoproteins in normal liver in vivo and in its tumor derivative in culture. What derangement may lead to the formation of an intracellular pool of perhaps fully assembled plasma membrane vesicles is unknown, but their apparent failure to become incorporated into the cell surface in a normal fashion may be related to the defects in the synthesis and secretion of proteins seen in most hepatoma cells. Further studies in both systems are needed before it can be determined how useful HTC and other cultured cells will be for the study of plasma membrane biogenesis in liver and other normal tissues.

Acknowledgment—I am greatly indebted to Mr. Milton Fisher for his excellent technical assistance during this work.

REFERENCES

Plasma Membrane Glycoprotein Biogenesis