# Localization and Biosynthesis of NADH-Cytochrome $b_5$ Reductase, an Integral Membrane Protein, in Rat Liver Cells

## III. Evidence for the Independent Insertion and Turnover of the Enzyme in Various Subcellular Compartments

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ABSTRACT   The biosynthesis and turnover of rat liver NADH-cytochrome $b_5$ reductase was studied in in vivo pulse-labeling and long-term, double-labeling experiments. Rats under thiopental anesthesia were injected into the portal vein with [$^3$H]L-leucine and sacrificed at various times after the injection. NADH-cytochrome $b_5$ reductase was extracted from liver cell fractions by cathepsin D-catalyzed cleavage and was then immunoadsorbed onto antireductase-bearing affinity columns in the presence of excess unlabeled rat serum. After elution of the enzyme from the columns with a pH-2.2 buffer, the amount of the reductase protein in the samples was determined by radioimmunoassay, and the radioactivity in reductase was determined on SDS polyacrylamide gel reductase bands. The specific radioactivity of the reductase extracted from the homogenate as well as from rough and smooth microsomal, mitochondrial, and Golgi fractions, estimated at the end of the pulse (10 min after the injection) and at various time points thereafter, remained approximately constant over a 6-h period. These data suggest that the enzyme is independently inserted into the various membranes where it is located. Moreover, the specific radioactivity of the mitochondrial reductase was lower than that of the other fractions, suggesting that it turns over at a slower rate. The lower turnover rate of the mitochondrial enzyme was confirmed by long-term, double-labeling experiments carried out according to the technique of Arias et al. (*J. Biol. Chem.* 244: 3303-3315.). The relevance of these findings in relation to the understanding of membrane biogenesis and turnover is discussed.

In the preceding articles of this series we demonstrated that NADH-cytochrome $b_5$ reductase has a multicompartment distribution, because high specific activities, not attributable to cross-contamination, were detected in microsomes (MR),[1] mi-

tochondria, and Golgi fractions (7). In addition, we provided evidence that in these various locations the activity is probably not due to isoenzymes but is accounted for by a single protein (27). In the present article, we report the results of our in vivo studies on the biosynthesis and turnover of this protein. We

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; ER, endoplasmic reticulum; GF$_{1+2}$ and GF$_3$, light and heavy Golgi fractions, respectively; MR, microsomes; PAGE, polyacrylamide gel electrophoresis; l-reductase, water soluble fragment of NADH-cytochrome

$b_5$ reductase cleaved by lysosomal enzymes; PBS, phosphate-buffered saline; TCA, trichloroacetic acid.

feel that biogenetic studies of a protein common to various membranes are of special interest because they can provide information concerning two important questions: (*a*) do rough endoplasmic reticulum (ER) membranes represent the precursors of other cellular membranes, and (*b*) is the turnover of membrane proteins determined exclusively by the physico-chemical properties of the protein, or is turnover also determined by the type of membrane in which the protein is located?

## MATERIALS AND METHODS

### General

Sucrose solutions and apparatus were the same as those described in the first article of this series (7). Enzyme assays, protein determination, and SDS polyacrylamide gel electrophoresis (SDS-PAGE) in 8 × 130-mm tubes were carried out as described in the second (27). Solutions indicated in abbreviated form are: PBS, phosphate-buffered saline; PBS-BSA, phosphate-buffered saline containing 0.3% bovine serum albumin.

### Cell Fractionation

Preparation of MR, mitochondria, and heavy and light (GF$_3$ and GF$_{1+2}$, respectively), and total Golgi fractions was carried out as described in the preceding articles in this series (7, 27).

To prepare rough and smooth MR, the material collected from the load zone of the discontinuous gradient after flotation of Golgi bands was mixed with 2.5 M sucrose to obtain a final concentration of 1.35 M and loaded in a discontinuous sucrose gradient consisting of the following layers: 2 ml of 2 M sucrose, 10 ml of load, and 6 ml of 1.3 M sucrose. All sucrose solutions were buffered at pH 7.4 with 3 mM imidazole-HCl. The tubes were filled with 0.4 M buffered sucrose and centrifuged at 25,000 rpm overnight (SW 27 rotor). The material floating above the 1.35 M sucrose layer was taken as smooth MR, and that at the 1.35–2 M sucrose interface as rough MR. The suspensions were diluted with 3 vol of 3 mM imidazole-HCl buffer, pH 7.4, and sedimented into pellets (42,000 rpm, 1 h, 60 Ti rotor).

### Radioactive Labeling In Vivo

PULSE LABELING: Male Sprague-Dawley rats weighing 200–230 g and starved overnight, were anesthetized by the injection of 6 mg thiopental into the caudal vein.[2] 1.5 ml of PBS containing [$^3$H]L-leucine in the amounts specified in the table and figure legends were injected into the portal vein over a period of 1 min. Animals were sacrificed at various times thereafter (see figure legends). For each time point, two animals were used.

LONG-TERM LABELING: To study the turnover of NADH-cytochrome $b_5$ reductase by the double-label technique of Arias et al. (1), two rats weighing ~170 g each were injected intraperitoneally with [$^3$H]L-leucine and then starved overnight. 6 d later, they were similarly injected with [$^{14}$C]L-leucine. A control rat was injected simultaneously with [$^3$H]- and [$^{14}$C]L-leucine. Radioactive tracer doses are specified in Table II. Control and experimental rats were sacrificed 12.5 h after the last injection.

### Isolation of Radioactive NADH-Cytochrome $b_5$ Reductase by Cathepsin D Treatment Followed by Immunoadsorption

For cathepsin D solubilization of NADH-cytochrome $b_5$ reductase (30), suspensions of cell fractions in 0.25 M buffered sucrose were mixed with $\frac{1}{10}$ their volume of a solution that contained Tris-maleate buffer, pH 5.5 (0.8 M), EDTA (8 mM) (17,000 U/ml), streptomycin (17 mg/ml), and cathepsin D (14 U/ml). The suspensions, whose final pH was 5.8, were incubated for 8 h at 37°C in a shaker bath and then neutralized by the addition of 1 M NaOH. The total homogenate was incubated under the same conditions, but the addition of cathepsin D was unnecessary. After ultracentrifugation of the digests (30,000

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[2] Thiopental was chosen on the basis of experiments in which we investigated the effect of various anesthetics on liver cell fractionation. We found that chloralose and ether increase the NADH-cytochrome *c* reductase activity up to fourfold in Golgi fractions without increasing protein recovery. In contrast, thiopental caused relatively minor alterations of the parameters investigated.

rpm, 40 min, 40 rotor), the supernates were found to contain ~90% of the recovered NADH-FeCN reductase activity.

The material released by cathepsin D digestion was processed by affinity chromatography as described in the preceding article in this series (27), but detergents were omitted from the solutions. Columns containing ~1 mg of antireductase antibody conjugated to Sepharose 4B were loaded with the supernates obtained from up to the following amounts of material: total homogenate, ~40 mg of protein; mitochondria, ~50 mg of protein; rough and smooth MR, ~30 mg of protein; total Golgi, ~10 mg of protein.

In pulse-labeling experiments, because of the high specific radioactivity of the secretory proteins, it was found necessary to add an excess of nonradioactive rat serum to the supernates obtained after cathepsin D digestion. Amounts of serum added, calculated to contain at least a fivefold excess over endogenous secretory protein, were, per milligram of protein of the initial cell fractions: smooth and rough MR, 36 μl; Golgi, 72 μl; mitochondria, 9 μl; total homogenate, 18 μl.

After elution of the immunoadsorbed material from the antibody-Sepharose conjugate with 3.0 ml of 0.2 M glycine-HCl buffer, pH 2.2, the columns were washed with 3 ml of PBS and then eluted again with 3.0 ml of the glycine-HCl buffer. The reductase-containing eluates were combined and brought to 7 ml with glycine-HCl buffer, and an aliquot (0.3 ml) was removed for radioimmunoassay. 100 μg of bovine serum albumin (BSA) and trichloroacetic acid (TCA), at a final concentration of 10%, were added to the remaining 6.7 ml. The samples were allowed to stand at 4°C overnight. Then they were centrifuged, and the precipitates were collected and processed for SDS-PAGE as previously described (27).

After use, affinity columns were washed extensively with PBS–0.02% NaN$_3$. Before being used again, 3 ml of 0.2 M glycine-HCl buffer, pH 2.2, was passed through the columns. No detectable radioactivity was eluted with this treatment.

### Determination of Radioactivity

TCA PRECIPITATES OF CELL FRACTIONS: Aliquots of cell fractions were precipitated with 10% TCA, washed twice with 5% TCA and then dissolved in Protosol and counted in toluene containing 2,5-diphenyloxazole and 1,4-bis[2-(5-phenyloxazolyl)]benzene, with an efficiency for $^3$H of 45%.

NADH-CYTOCHROME $b_5$ REDUCTASE: In pulse-labeling experiments, the material eluted from the affinity columns (see above) was electrophoresed on 10% SDS polyacrylamide gels made up in 8 × 130-mm tubes. After staining with Coomassie Brilliant Blue, a 2-mm slice centered on the reductase band was cut out. Three 2-mm slices behind this band and two ahead of it were also cut. After the slices were dried, solubilization and bleaching of each slice were accomplished by incubation with 0.3 ml of 30% H$_2$O$_2$ for 2 h at 45°C in tightly closed glass vials. After the addition of 10 ml of Lumagel, samples were counted four times for 40 min each time. The efficiency for $^3$H was ~40%. The radioactivity in reductase was taken as the sum of the counts in the reductase-centered slice and the two neighboring slices. The background was taken as the average of the two slices adjacent to these three slices. Values found in these two slices were close to the general background of the gel. Examples of radioactivity distribution in gel slices are shown in Fig. 3.

In the double-labeling experiments, gels were sliced and processed in the same way as described above. Efficiency for $^{14}$C, $^3$H, and $^{14}$C spill in the $^3$H channel, determined by internal standardization, were ~55, 25, and 8%, respectively.

### Estimation of NADH-Cytochrome $b_5$ Reductase in Immunoeluates

Two methods were used.

RADIOIMMUNOASSAY: $^{125}$I-1-reductase was prepared by the chloramine T method (19). To eliminate aggregates formed during the iodination procedure, the samples were subjected to Sephadex G 100 chromatography on a 1 × 20 cm column equilibrated in PBS-BSA. Nonaggregated reductase was then purified by affinity chromatography using antireductase–Sepharose 4B conjugate. The eluted material was neutralized by the addition of 1 M K$_2$HPO$_4$ (in a 1:3.5 ratio to the sample in 0.2 M glycine-HCl, pH 2.2), concentrated to 1.3 × 10$^6$ cpm/ml with an ultrafiltration apparatus (PM 10 filter) (Amicon Corp., Scientific Sys. Div., Lexington, Mass.), and stored at −20°C in small aliquots. Over 90% of the $^{125}$I-antigen prepared in this way was found to be immunoprecipitable.

Samples to be assayed were neutralized with 1 M K$_2$HPO$_4$ supplemented with BSA (0.3% final concentration) and stored at −20°C until used. Assay mixtures contained: 50 μl of standard (purified 1-reductase [27]) or unknown sample in K$_2$HPO$_4$ (0.255 M), glycine (0.15 M), and HCl, pH 7; EDTA, 3 mM; BSA, 0.3%; $^{125}$I-1-reductase, specific activity 2.6 × 10$^5$ cpm/μg, 12,000 cpm; and antireductase antibody, purified by affinity chromatography (27), 17 ng, to a final volume of 120 μl. The relative proportion of $^{125}$I-antigen and antibody was chosen to yield a 25–30% radioactivity binding in the absence of unlabeled antigen. Incubations

were carried out in siliconized Pyrex tubes coated with BSA for 24 h at 4°C. 20 µl of a 1:15 dilution of nonimmune rabbit serum and 40 µl of goat antirabbit serum were then added. After another 24 h of incubation, samples were diluted with 2 ml of PBS-BSA and centrifuged (1,000 $g_{av}$, 15 min). The pellets were resuspended in 1 ml of PBS-BSA and quantitatively transferred to fresh test tubes (two 0.5-ml washes). Much of the background due to sticking of the iodinated protein to the test tubes could be eliminated in this way. After recentrifugation, the immunoprecipitates were counted in a Packard Autogamma type 5110 spectrophotometer (Packard Instrument Co., Inc., Downers Grove, Ill.). An example of a radioimmunoassay standard curve is shown in Fig. 4. Routinely, three different quantities of reductase-containing samples were assayed each in triplicate.

QUANTITATIVE MICRODENSITOMETRY OF COOMASSIE BRILLIANT BLUE STAINED POLYACRYLAMIDE GELS: TCA precipitates of the immuno-eluates were resolved in cylindrical polyacrylamide gels, as described above, and stained with Coomassie Brilliant Blue. In each assay, a standard curve was constructed by processing in parallel several aliquots of purified 1-reductase. Gels were scanned at 620 nm in a Joyce-Loebl MK 11 microdensitometer (Joyce, Loebl and Co., Ltd., Gateshead-on-Tyne, England), with a gel to tracing ratio of 1:9. The reductase peaks were cut out of the tracings and their areas estimated gravimetrically. Under our conditions, the standard curves were linear between 10 and 50 µg of applied 1-reductase.

## Materials

In addition to those listed in the previous articles in this series (7, 27), the following reagents were purchased from the indicated sources: L-[3,4-$^3$H]leucine (46 Ci/mmol) and L-[U-$^{14}$C]leucine (350 mCi/mmol), the Radiochemical Centre, Amersham, England; L-[U-$^{14}$C]leucine (280 mCi/mmol) and Na$^{125}$I, CIS Sorin, Saluggia, Italy; bovine spleen cathepsin D, Sigma Chemical Co., St. Louis, Mo.; thiopental, Farmitalia, Milan, Italy; Lumagel, Lumac System AG, Basel, Switzerland.

Antirabbit γ-globulin serum was produced in goats injected repeatedly with rabbit IgG purified by (NH$_4$)$_2$SO$_4$ precipitation followed by diethylaminoethyl cellulose column chromatography.

## RESULTS

### Pulse-labeling Experiments

INCORPORATION OF RADIOACTIVITY INTO THE TOTAL HOMOGENATE AND CELL FRACTIONS: To establish the kinetics of [$^3$H]L-leucine incorporation into hepatic TCA-precipitable material in the intact animal, a preliminary experiment was carried out in which rats were sacrificed at various times after the beginning of the injection (Fig. 1). Incorporation of the tracer was found to proceed linearly for 8–10 min, after which time ~8% of the injected counts were precipitated by TCA from the liver homogenate. Therefore, in subsequent experiments, the 10-min time point was chosen as the earliest to be examined.

Fig. 2 illustrates the changes in specific radioactivity of TCA precipitates obtained from total homogenates and from five cell fractions (rough and smooth MR, mitochondria, GF$_3$, and GF$_{1+2}$) 10–360 min after injection of [$^3$H]L-leucine into the portal vein. The highest value in rough MR was found at the first time point, whereas an increase was observed in smooth MR, GF$_3$, and GF$_{1+2}$ at the 25-min point. Especially striking was the enrichment in radioactive proteins in GF$_{1+2}$ (nearly 20-fold over the homogenate). Thereafter, the radioactivity decreased in these four fractions. These kinetics of labeling are those expected for the cell fractions derived from successive compartments along the secretory pathway and agree well with the data published by others (3). In contrast, the mitochondrial fraction was very poorly labeled and its specific radioactivity remained nearly constant during the time period studied.

EXTRACTION OF NADH-CYTOCHROME $b_5$ REDUCTASE FROM CELL FRACTIONS AND DETERMINATION OF ITS SPECIFIC RADIOACTIVITY: Reductase was solubilized by cathepsin D cleavage. This procedure had the advantage of
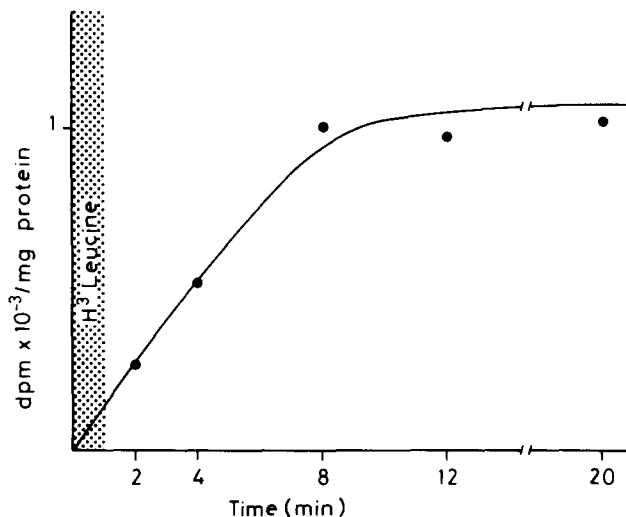


FIGURE 1  Incorporation of radioactivity into TCA-precipitable material in rat liver after a pulse of [$^3$H]L-leucine injected into the portal vein. Rats, anesthetized with thiopental (34 mg/kg body wt), each received an injection of [$^3$H]L-leucine (4 µCi/100 g body wt in 1.5 ml of PBS) into the portal vein. The animals were sacrificed at the times indicated and liver homogenates were prepared and used for protein and radioactivity determinations. Each value given represents the average from two animals. The stippled area indicates the duration of the [$^3$H]L-leucine injection.

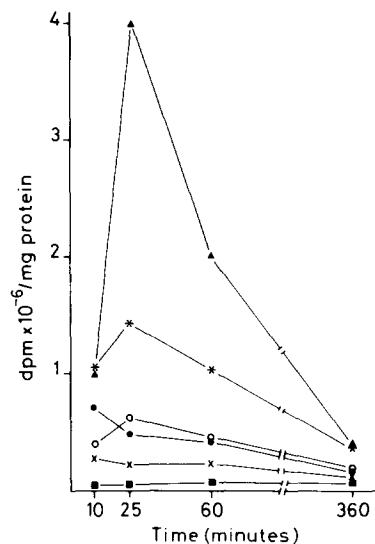

FIGURE 2  Incorporation of radioactivity into TCA-precipitable material of various cell fractions prepared from liver homogenates after an in vivo pulse of [$^3$H]L-leucine injected into the portal vein. Eight rats, anesthetized with thiopental (36 mg/kg body wt), each received an injection of [$^3$H]L-leucine (1.5 mCi in 1.5 ml of PBS) into the portal vein. Two animals were sacrificed at each time point indicated on the abscissa. The livers were pooled and homogenized, cell fractions were prepared, and the specific radioactivity of TCA-precipitable material was determined. (×) Total homogenate. (●) Rough MR. (○) Smooth MR. (■) Mitochondria. (*) GF$_3$. (▲) GF$_{1+2}$.

allowing a partial purification of the reductase before it was processed by immunoadsorption and also of avoiding the presence of detergents during purification. After incubation with cathepsin D at pH 5.8 for 8 h, ~90% of the NADH-FeCN reductase activity was recovered in the high-speed supernate of all cell fractions investigated. However, an exact quantitative estimate of the solubilization could not be made because

considerable inactivation of the enzyme did result from the incubation at low pH (~50% in 8 h, not shown).

After being processed by immunoadsorption, the reductase-containing samples were analyzed by SDS-PAGE. However, at the early time points, the radioactivity background on the gel was high, presumably because of the presence of rapidly turning over, contaminating secretory proteins or protein fragments. To overcome this difficulty, an excess of nonradioactive rat serum was added to the cathepsin D solubilized samples to compete with the labeled secretory proteins for binding sites on the affinity columns. This treatment was found to be very effective, and symmetrical, reproducible radioactivity peaks centered on the reductase band were obtained (Fig. 3).

To obtain values for the specific radioactivity of reductase, the amount of reductase protein loaded onto the gels was determined. In the gels of this experiment, in which samples had been supplemented with nonlabeled rat serum, the staining background was considerable, and accurate measurements by microdensitometry of the reductase were thus impossible. These measurements were therefore carried out by radioimmunoassay of the material eluted from the immunoaffinity columns. Fig. 4 illustrates a typical radioimmunoassay standard curve, which shows that reliable data could be obtained over a range of from 5 to 30 ng of reductase.

Specific radioactivities of NADH-cytochrome $b_5$ reductase extracted from the total homogenate, obtained as described above, are shown in Fig. 5. In contrast to the behavior observed for total proteins of the homogenate (Fig. 2), in which the TCA-precipitable specific radioactivity decreased to about one-half its initial value after 6 h, the labeling of the reductase remained constant over the time period studied. Therefore, the results shown in Fig. 5 demonstrate that the analysis of the labeling kinetics of the reductase band carried out in this way was not affected by the presence of secretory proteins.
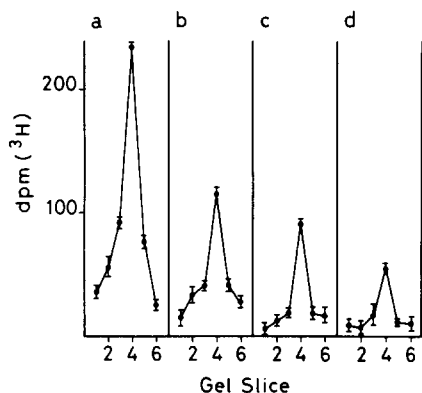


FIGURE 4  Typical standard curve of the radioimmunological estimation of NADH-cytochrome $b_5$ reductase. Values in abscissa refer to amounts of purified I-reductase standard added to the incubation mixture.



FIGURE 5  Specific radioactivity of NADH-cytochrome $b_5$ reductase isolated from the total liver homogenate at various times after a pulse of [$^3$H]L-leucine injected into the portal vein. NADH-cytochrome $b_5$ reductase was extracted from total homogenates of the experiment illustrated in Fig. 2 by lysosomal digestion and then purified by immunoadsorption (in the presence of excess unlabeled rat serum), followed by SDS-PAGE. The specific radioactivity was determined by scintillation counting of the band separated in the gel and by radioimmunoassay of the reductase protein. Values given are the average of two separate determinations carried out on two aliquots of total homogenate for each time point. Bars represent standard errors.

The data for cell fractions are given in Table I. Because in the case of Golgi fractions the material was insufficient for two separate specific radioactivity determinations, the standard deviations given in Table I have been calculated on the basis of repeated counts and radioimmunoassays of one sample. When separate determinations were carried out (mitochondria, MR, total homogenate) the values fell well within the standard deviations given in Table I (see, for instance, Fig. 5). Variance analysis of these data demonstrated that differences among time points were not significant, whereas those among fractions were highly significant ($P < 0.01$). The latter could be attributed exclusively to the difference between mitochondria and the other cell fractions, as demonstrated by Student's $t$ tests among couples of fractions averaged over the four time points. Differences among smooth and rough MR and Golgi fractions were not significant. Thus, the following conclusions can be drawn: (a) in each fraction the specific radioactivity of NADH-



FIGURE 3  SDS-PAGE analysis of NADH-cytochrome $b_5$ reductase-containing samples extracted from cell fractions at various times after a pulse of [$^3$H]L-leucine was injected into the portal vein. NADH-cytochrome $b_5$ reductase was extracted from the cell fractions illustrated in Fig. 2 by cathepsin D digestion and then immunoadsorbed onto antireductase-bearing affinity columns in the presence of excess unlabeled rat serum (see Materials and Methods for details). After elution from the columns, the samples were fractionated by SDS-PAGE, and the distribution of radioactivity determined in gel slices. The scans shown correspond to reductase bands derived from: (a) rough MR at the 10-min time point; (b) smooth MR at the 25-min time point; (c) mitochondria at the 60-min time point; (d) total Golgi fraction at the 6-h time point. Slice 4 was centered on the reductase band revealed by Coomassie Brilliant Blue staining. Bars indicate standard deviations for the values averaged over four separate 40-min counting periods. Quantities of reductase measured by radioimmunoassay were: a, 20.4 μg; b, 8.4 μg; c, 15.2 μg; d, 5.1 μg.

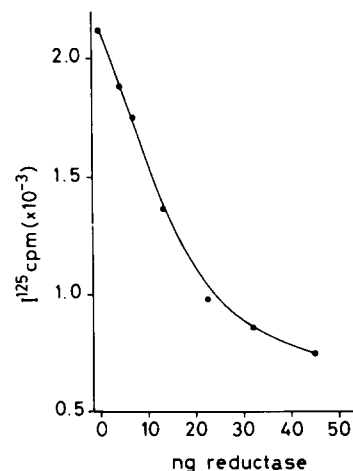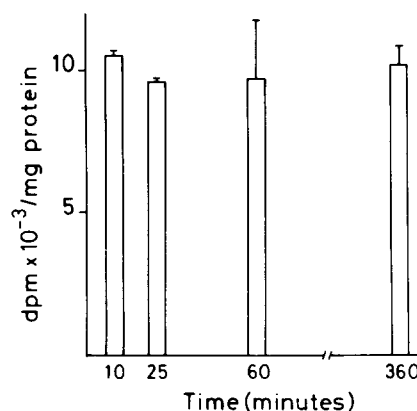cytochrome $b_5$ reductase remained constant over the time period studied; (b) no significant differences among rough MR, smooth MR, and Golgi could be detected at any of the time points analyzed; (c) the reductase extracted from mitochondria had a specific radioactivity lower than that of the other cell fractions at all time points, suggesting the possibility of a lower degradation rate of the enzyme in this compartment.

## Long-term Labeling Experiments

Further information on the biosynthesis and turnover of NADH-cytochrome $b_5$ reductase in its various subcellular locations was obtained by long-term experiments carried out in unanesthetized rats by the double-label technique of Arias et al. (1). 6 d after an injection of [$^3$H]L-leucine, the animals received [$^{14}$C]L-leucine and were sacrificed 12.5 h later. The reductase was extracted and purified from cell fractions in the same way as in the pulse-labeling experiments (but without the addition of nonradioactive rat serum), and the $^{14}$C:$^3$H ratio was determined (Table II). As can be seen from the values found in the controls, which were injected simultaneously with the $^{14}$C- and $^3$H-labeled amino acid, the reproducibility of the radioactivity determination was good. In two separate experiments we found that the $^{14}$C:$^3$H ratios of the reductase purified from microsomes, rough and smooth, as well as from the Golgi fraction, were similar, whereas those of the mitochrondrial enzyme were considerably lower. In experiment 2 of Table II,

also illustrated in Fig. 6, the specific $^{14}$C radioactivity of the reductase in its various locations was also calculated, using microdensitometry to estimate the amount of the enzyme in gel bands. Again, no significant differences emerged among MR and Golgi fractions, whereas labeling in the mitochondrial reductase was 50–60% of that of the other fractions. The findings reported in this section confirm, therefore, that rat liver NADH-cytochrome $b_5$ reductase turns over more slowly in mitochondria than in the other subcellular compartments where it is located.

## DISCUSSION

Several technical difficulties were encountered during the present work. Because of the slow turnover of membrane proteins, it was necessary to use very large amounts of labeled precursors (12 mCi of [$^3$H]L-leucine per pulse-labeling experiment). Even with these massive amounts, the specific radioactivity of NADH-cytochrome $b_5$ reductase was low, and, considering the small size of the Golgi compartment and the fact that the reductase is a minority component in membranes, the total amounts of radioactivity with which we had to work were indeed very small. Therefore, it was crucial to ensure that our analyses would not be impaired by the presence of contaminating secretory proteins, whose specific radioactivities are over 100 times higher than that of reductase. To this end, it was not sufficient to determine the radioactivity of the reductase band

TABLE I

Specific Radioactivity of NADH-Cytochrome $b_5$ Reductase * Isolated from Rat Liver Cell Fractions at Various Times after a Pulse of [$^3$H]L-Leucine‡ Injected into the Portal Vein§

| Cell fraction | Time | | | |
| --- | --- | --- | --- | --- |
| | 10 | 25 | 60 | 360 |
| | min | | | |
| Rough MR | 13,180 ± 2,670 | 10,840 ± 2,550 | 11,010 ± 1,160 | 15,210 ± 1,720 |
| Smooth MR | 11,480 ± 550 | 12,600 ± 1,370 | 13,880 ± 540 | 15,110 ± 1,150 |
| Total golgi | 11,710 ± 2,010 | | 14,380 ± 2,360 | 12,690 ± 2,300 |
| Mitochondria | 5,460 ± 1,700 | 6,000 ± 1,600 | 5,730 ± 1,210 | 5,720 ± 990 |

* NADH-cytochrome $b_5$ reductase was extracted from cell fractions and purified, and its specific radioactivity was determined as described in the legend of Fig. 5.
‡ 0.75 mCi/100 g body wt.
§ Values of one experiment. Standard deviations were calculated on the basis of variability in counting and in radioimmunoassay determinations.

TABLE II

Relative Turnover of NADH-Cytochrome $b_5$ Reductase in Rat Liver Cell Fractions

| Cell fraction | Experiment 1 | | Experiment 2 | | |
| --- | --- | --- | --- | --- | --- |
| | Control* | Experimental‡ | Control* | Experimental‡ | |
| | $^{14}$C:$^3$H§ | $^{14}$C:$^3$H§ | $^{14}$C:$^3$H§ | $^{14}$C:$^3$H§ | $^{14}$C |
| | | | | | dpm/mg protein‖ |
| Total homogenate | 0.150 | 0.213 ± 0.004 | — | 0.433 ± 0.011 | 4,860 ± 640 |
| Rough MR | 0.150 | 0.239 ± 0.011 | 0.269 | 0.452 ± 0.012 | 4,910 ± 70 |
| Smooth MR | — | — | 0.244 | 0.383 ± 0.029 | 5,580 ± 1,200 |
| GF$_3$ | — | 0.233 | — | — | — |
| Total Golgi | — | — | — | 0.422 | 4,680 |
| Mitochondria | 0.150 | 0.171 ± 0.019 | 0.246 | 0.267 ± 0.002 | 3,180 ± 430 |

* A control rat was injected intraperitoneally simultaneously with $^3$H and [$^{14}$C]L-leucine 12.5 h before sacrifice. Doses injected were 800 and 125 μCi, respectively, in experiment 1 and 580 and 150 μCi, respectively, in experiment 2.
‡ Two rats were injected intraperitoneally with [$^3$H]L-leucine and with [$^{14}$C]L-leucine 6 d later. They were sacrificed 12.5 h after the second injection. Doses injected were 2 and 0.30 mCi, respectively, in experiment 1 and 1.15 and 0.3 mCi, respectively, in experiment 2.
§ $^{14}$C:$^3$H ratios were determined on the l-reductase bands of SDS polyacrylamide gels of immunoeluates. Standard errors are given for samples on which two separate determinations were carried out.
‖ l-Reductase was measured by quantitative microdensitometry of the corresponding band in SDS polyacrylamide gels stained with Coomassie Brilliant Blue. Standard errors are given for samples on which two separate determinations were carried out.
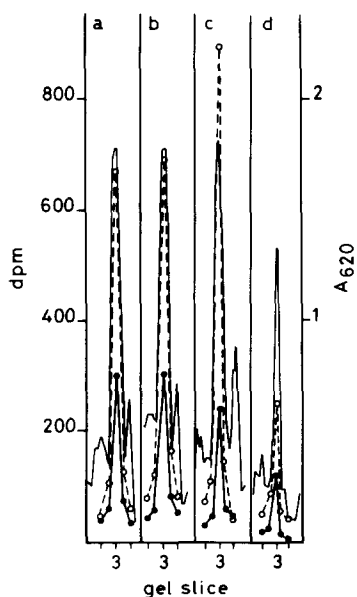
FIGURE 6   SDS-PAGE analysis of NADH-cytochrome $b_5$ reductase-containing samples extracted from various cell fractions obtained from rats injected with [$^3$H]L-leucine and then with [$^{14}$C]L-leucine 6 d later (experimental animals of experiment 2, Table II). NADH-cytochrome $b_5$ reductase was extracted from cell fractions and purified by immunoadsorption without added unlabeled rat serum (see Materials and Methods). The material eluted from the affinity columns was electrophoresed on SDS polyacrylamide tube gels, which were stained with Coomassie Brilliant Blue and then scanned at 620 nm with a gel to tracing ratio of 1:1 (—). $^{14}$C (●) and $^3$H (○) radioactivity were determined on 2-mm gel slices. The scans shown correspond to: (a) total homogenate; (b) rough MR; (c) mitochondria; (d) total Golgi (GF$_{1+2+3}$). Slice 3 was centered on the reductase band.

separated from the contaminating proteins of the immunoeluates by SDS-PAGE because the background of the gel was high, and because the apparent specific radioactivity of the reductase decreased with time much more rapidly than would have been expected for a nonsecretory protein (data not shown). This result was surprising because, as reported in the previous article in this series (27), no contaminants were detected by peptide mapping analysis in the reductase preparations obtained in this way. Clearly, however, the peptide mapping technique is inadequate to reveal trace amounts of contaminants which, because of their high specific radioactivity, were sufficient to affect our pulse-labeling analyses. Fortunately, this problem could be overcome by adding an excess of cold, competing rat serum to the cathepsin D-extracted material before processing by immunoadsorption. In the long-term labeling experiments, in which animals were sacrificed 12.5 h after tracer injection, the specific radioactivity of intracellular secretory proteins was low, and, therefore, the addition of rat serum to the immunoeluates was unnecessary.

A second problem concerned the determination of the amounts of reductase contained in the bands we analyzed. One possibility we considered was to measure the NADH-FeCN reductase activity of the material eluted from the affinity columns. However, this approach was discarded because the long incubation at 37°C and pH 5.8 with cathepsin D necessary to detach the enzyme from membranes effected a considerable inactivation of the activity, and this inactivation was found to be unequal in the various cell fractions (not shown). A second

possibility was to determine the areas of the Coomassie Blue-stained reductase bands after scanning of the gels. This approach, however, was entirely adequate only for the samples of the long-term labeling experiments, whereas, in pulse-labeling samples, because of the presence of excess unlabeled serum proteins, the staining background on the gels was considerable, and, therefore, the measurement of the area corresponding to the reductase band could only be approximate. We therefore turned to radioimmunoassay, which gave us satisfactory results. A valid objection to this method for the determination of specific radioactivity is that reductase *protein* was measured in immunoeluates from affinity columns, whereas reductase *radioactivity* was measured two steps further, i.e., after TCA precipitation and SDS-PAGE. However, the possibility that our results were substantially affected by incomplete recovery is unlikely because the low specific radioactivity observed in mitochondrial reductase after pulse-labeling was confirmed in long-term labeling experiments on the basis of both $^{14}$C:$^3$H ratios and specific radioactivity measurements in which protein estimation was carried out by a different method, i.e., quantitative microdensitometry. We therefore feel confident in drawing two main conclusions from our work: (a) with a 10-min pulse of [$^3$H]L-leucine, no transport of the enzyme from the rough ER to other cell compartments could be detected, and (b) the half-life of NADH-cytochrome $b_5$ reductase is of the order of days and is longer in mitochondria than in the other cell fractions.

Although double-label isotope ratios have been used to calculate half-lives of liver proteins (17), it is clear that, particularly in the case of slowly turning over proteins, reutilization problems are important, and, therefore, calculations of absolute rates require the knowledge of the labeling kinetics of the precursor pool (38). Our long-term labeling data were, therefore, used for comparative purposes only.

The conclusions that we have drawn are at variance with those recently reached by Okada and Omura (30) who reported that mitochondrial and MR reductases are characterized by the same turnover, and that the reductase appears first in rough ER, to then be transported to smooth ER and mitochondria. However, upon close analysis of the data of these authors, it appears that their conclusions can be questioned. In fact, the conclusion that mitochondrial and MR reductases have the same turnover was reached on the basis of the interpretation that the same line fits the data for the two reductases in a semilogarithmic plot relating the specific radioactivity of the enzyme to time after a single injection of [$^3$H]L-leucine (30). However, when we recalculated the data of Okada and Omura (30) using the least square method to obtain the best linear fit, different lines were obtained, with a slower apparent turnover for the mitochondrial enzyme. With regard to their in vivo pulse-labeling experiments (30), it must be stated that at all points analyzed the specific radioactivity of the mitochondrial reductase was lower than that of the enzyme in smooth and rough MR fractions. The only indication of a reductase transport from rough ER to other compartments was a transient increase in the rough MR specific radioactivity at 15 and 30 min. However, because the measurements were based on values found in immunoprecipitates of only partially purified reductase preparations, it cannot be excluded that the data of the early time points were influenced by contaminating secretory proteins.

We now turn to the interpretation of our results in terms of biogenetic relationships among membranes. Our pulse-labeling

data indicate that either NADH-cytochrome $b_5$ reductase is independently inserted into its various membrane locations, or that transport of newly synthesized molecules among membrane-bounded compartments is so rapid as to be complete within a few minutes (a time shorter than 10 min, the duration of our pulse). Because the half-life of the reductase is of the order of days (this paper and reference 30), such a rapid transport could not be unidirectional, unless "new" molecules were segregated from "old" molecules and transported preferentially. On the other hand, a continuous exchange of reductase molecules among the various membranes is not feasible for the mitochondrial compartment, where reductase turns over more slowly than in the other organelles. Furthermore, also in the ER and Golgi complex, the rate of equilibration is unlikely to be fast enough to account for our results inasmuch as the available data on equilibration of proteins between rough and smooth ER membranes indicate that the time involved is much longer. For example, newly synthesized cytochrome $P_{450}$ has been found to equilibrate within one h, and not within a few min (15). Thus, the first interpretation of our results, i.e., that reductase is inserted independently into different membranes, is the more likely one, although a definite answer would require an experimental system in which shorter incorporation pulses are feasible.

The conclusion that NADH-cytochrome $b_5$ reductase is inserted directly into different membranes is consistent with the orientation of this protein as well as with its physicochemical properties. In fact, the reductase is probably an endoprotein inasmuch as it is exposed at the cytoplasmic face of membranes (6, 10), and the purified uncleaved enzyme extracted from MR by detergents is capable of reinserting into MR membranes (36).[3] Moreover, recent data indicate that the hydrophobic membrane binding segment of NADH-cytochrome $b_5$ reductase is located toward the C terminus of the molecule (28). Thus, the affinity of this protein for membranes is a property that should appear when synthesis is complete (or nearly complete). Therefore, its insertion into membranes, in contrast to the mechanism of other integral membrane proteins (12, 22, 24), might not necessarily be contranslational but might possibly be posttranslational. Recent results of studies in a cell free system (5) demonstrate that NADH-cytochrome $b_5$ reductase is synthesized on free polyribosomes. It is of interest that cytochrome $b_5$, which is similar in many respects to NADH-cytochrome $b_5$ reductase, has also been found to be synthesized on free polyribosomes (31, 33). In contrast, another membrane protein, also probably restricted to the cytoplasmic surface, NADPH-cytochrome $P_{450}$ reductase, has been reported to be synthesized on bound polyribosomes (31).

Because NADH-cytochrome $b_5$ reductase is synthesized on free polyribosomes, it could possibly move through the cytoplasm before reaching its membrane destinations. In our pulse-labeling experiments, we checked for the possibility of a soluble precursor pool of reductase and found no evidence for its existence (not shown). However, because membrane endoproteins made on free polyribosomes have been found to have transit times through the cytoplasm as short as 2 min (2), such a precursor pool could have gone undetected in our experiments.

[3] This insertion should not be considered as a simple partition into a lipid phase because recent data (14) demonstrate that both NADH-cytochrome $b_5$ reductase and cytochrome $b_5$ binding to MR is tighter than the binding to liposomes, so that intervesicle exchange through the aqueous phase, previously demonstrated in the latter condition, is most probably impossible in the living cell.

If integral endoproteins, like NADH-cytochrome $b_5$ reductase, are inserted into membranes directly from the cytoplasm, interesting questions arise as to what determines the type of membrane into which these proteins become inserted and what determines their concentration in different organelles. For example, the concentration of cytochrome $b_5$ in liver plasma membrane is low (20), if it is present at all, and much of the cytochrome $b_5$-like activity of mitochondrial outer membrane is due to a protein different from microsomal cytochrome $b_5$ (16). Inasmuch as cholesterol has been reported to inhibit the binding of cytochrome $b_5$ to biological and artificial membranes (13, 37), it is possible that the membrane lipid composition influences the subcellular distribution of endoproteins such as NADH-cytochrome $b_5$ reductase. Alternatively, the restriction of endoproteins to a limited number of membrane types could depend not on a physicochemical property of these proteins but, rather, on a posttranslational modification carried out in only some membranes. A similar suggestion has been made by Rothman and Lenard (34).

Another finding of our study is the slow turnover of the reductase in mitochondria compared with its turnover in the other cell compartments. If the mitochondrial enzyme activity is attributable to the same protein molecule as that of MR and the Golgi complex, as suggested by the results reported in our previous article (27), it would appear that the same protein could be characterized by a different turnover rate in various subcellular locations. This would demonstrate that, in addition to molecular properties such as size, isoelectric point (18), glycosylation (25), and in vitro thermal stability (26), the physical environment of a protein contributes to its rate of degradation. Recent data indicate that at least two different mechanisms account for intracellular protein turnover: autophagy by lysosomal enzymes and an extralysosomal degradation whose rate differs for different proteins (9, 11, 32). Whether the difference in reductase turnover rate between mitochondria and Golgi-MR results from the differential contribution of these two mechanisms remains to be elucidated. On the other hand, because our findings on the molecular identity of NADH-cytochrome $b_5$ reductase in its different locations (27) concern mainly the hydrophylic portion of the enzyme, we cannot exclude that a subtle difference in the hydrophobic membrane-binding piece is responsible for the slower degradation rate of the mitochondrial reductase.

Finally, a few words on the general significance of our results in terms of models for membrane biogenesis. According to the "membrane flow" hypothesis (29), smooth ER, Golgi, and plasma membranes derive from the rough ER membranes by successive steps of transformation ("differentiation") and flow. Therefore, according to this model, constituents common to the membranes of the secretory pathway must derive from the rough ER, while components characteristic of membranes bounding compartments downhill on the secretory pathway might be added from the cytoplasm or originate from the transformation of rough ER constituents. Clearly, our findings, which strongly suggest that a common protein constituent is inserted independently in rough and smooth ER as well as in Golgi membranes, are incompatible with this model of membrane biogenesis. The membrane flow model should, however, not be confused with the idea that some, or many, membrane proteins must move through the rough ER to reach their final destination, as has been demonstrated in the case of vesicular stomatitis virus G protein (2, 22, 23), glycophorin A (21), and cytochrome $P_{450}$ (15). This is probably the case for many ectoproteins, i.e., proteins exposed at the lumenal or external

surface of membranes, which make use of the ribosome-membrane junction for their cotranslational transmembrane transfer (2, 4, 8, 12, 22, 24). Our findings, however, rule out the possibility that all integral membrane proteins are synthesized by bound polyribosomes and move through the rough ER (35) and can be added to the increasing evidence in favor of multiple mechanisms for membrane biogenesis.

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