ENDOPLASMIC RETICULUM MARKER ENZYMES IN GOLGI FRACTIONS--
WHAT DOES THIS MEAN?

KATHRYN E. HOWELL, AKIO ITO, and GEORGE E. PALADE. From the Section of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510. Dr. Ito's present address is the Department of Biology, Faculty of Science, Kyushu University, Fukuoka 812, Japan.

ABSTRACT

NADPH cytochrome c (cyt c) reductase and glucose-6-phosphatase, two enzymes thought to be restricted to the endoplasmic reticulum (ER) and widely used as ER markers, are present in isolated Golgi fractions assayed immediately after their isolation. Both enzymes are rapidly inactivated in fractions stored at 0°C in 0.25 M sucrose, conditions which do not affect the activity of other enzymes in the same preparation. The inactivation process was shown to be dependent on time and protein concentration and could be prevented by EDTA and catalase. Morphological evidence shows that extensive membrane damage occurs parallel with the inactivation. Taken together with the immunological data in the companion paper, the findings indicate that the enzymes NADPH cyt c reductase and probably glucose-6-phosphate are indigenous components of Golgi membranes.

KEY WORDS marker enzymes Golgi fraction microsomal fraction glucose-6-phosphatase NADPH cytochrome P₄₅₀ reductase enzyme inactivation

It is already established that the Golgi complex functions in the processing of secretory proteins in a wide variety of animal cells (10, 20). In addition, it has been postulated that it plays a role in membrane biogenesis as an intermediary station where membrane is modified while in transit from the endoplasmic reticulum (ER) to the plasma-lemma (16). An inquiry into this possible biogenetic role requires reliable data on the composition of the corresponding membranes, and especially on the existence, diversity, and extent of compositional overlap among them. Pertinent data, derived primarily from biochemical analyses of microsomal (ER) and Golgi fractions, indicate that the corresponding membranes are different, each possessing its own characteristic set of "marker" enzymes while sharing in different proportions common enzymatic activities. Glucose-6-phosphatase (G-6-Pase), NADPH-cytochrome P₄₅₀ (cyt P₄₅₀) reductase, and cytochrome P₄₅₀ are considered typical ER marker enzymes, their presence in Golgi fractions usually being ascribed to contaminating microsomes (3, 8, 17).

Recently we have found that G-6-Pase and NADPH cyt c reductase activities are significantly higher than originally reported if assayed immediately after the isolation of Golgi Fractions prepared by the procedure of Ehrenreich et al. (6). Both enzymes are rapidly inactivated under storage conditions which do not affect other enzymatic activities of the Golgi fraction. Since G-6-Pase and NADPH cyt c reductase are generally assumed to be restricted to ER membranes, we felt it was important to determine whether these activities are a result of bona fide Golgi components or are the result of increased contamination of Golgi fractions by ER-derived vesicles.

In this paper the inactivation process is described in some detail, while in the companion paper (9) immunological evidence is presented which leads us to conclude that morphologically identifiable Golgi elements have NADPH cyt c reductase activity, and probably also G-6-Pase activity.

MATERIALS AND METHODS

Materials

Enzyme substrates and specific biochemical com-
pounds were purchased from Sigma Chemical Company, St. Louis, Mo. Uridine diprophosphate galactose \([\text{galactose-1-}^{3}H(N)]\) 1-5 Ci/mmol was purchased from New England Nuclear, Boston, Mass.

**Animals**

All experiments were performed on 110-160 g male, Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.), starved overnight. When ethanol-treated, they were given 0.6 g ethanol/100 g body weight in a 50% solution by stomach intubation and were sacrificed by decapitation 90 min later.

**Cell Fractionation Procedures**

The Golgi Fraction \(1+2\) (GF\(_{1+2}\)) and residual microsomal fraction (RdMF) were isolated modifications of the procedure of Ehrenreich et al. (6).

**Homogenization:** The livers were collected in ice-cold 0.25 M sucrose, finely minced, forced through a tissue press of 1-mm mesh, and then homogenized in 0.25 M sucrose with 6-8 strokes of a motor-driven teflon pestle at 2,850 rpm in a glass homogenizer (no. C, A. H. Thomas Co., Philadelphia, Penn.) to give a 20% (wet wt of liver/vol) homogenate. The entire procedure was done at 4°C.

**Preparation of GF\(_{1+2}\) and RdMF**

All centrifugations were carried out in a Beckman L5-65 centrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). The homogenate was cleared of cell debris, nuclei, and mitochondria by centrifugation for 10 min at 10,000 \(g_{av}\), in a 60 Ti rotor. The resulting supernate was collected, diluted back to the original volume with 0.25 M sucrose and centrifuged in the same rotor for 90 min at 105,000 \(g_{av}\), to give microsomal pellets which were resuspended in 0.25 M sucrose in the original homogenizer with 3-5 strokes at the same speed as used initially. This suspension was mixed with 2.0 M sucrose to give a final refractive index of 1.3920, equivalent to that of 1.22 M sucrose (measured on a Bausch & Lomb refractometer, Bausch & Lomb Inc., Rochester, N. Y.). The final tissue equivalent was 1.5 g wt liver/ml. Then 10 ml of this total microsomal suspension was loaded under a discontinuous sucrose density gradient with 8-ml steps of 1.15 M, 0.86 M, and 0.25 M sucrose. The gradient was centrifuged in a SW 27 rotor (Beckman Instruments) for 180 min at 82,500 \(g_{av}\). The material that floated to the 0.25 M/0.86 M interface was collected and designated GF\(_{1+2}\). The material that remained in the most dense sucrose layer, including the portion that pelleted, was rehomogenized, diluted back to 0.25 M sucrose and designated RdMF.

**Electron Microscopy**

Aliquots of fractions (0.4 ml) were mixed with an equal volume of 4% OsO\(_4\) in water in 0.8-ml centrifuge tubes. After \(-2\) h, the suspensions were centrifuged in a SW 50.1 rotor for 20 min at 37,500 \(g_{av}\), and the pellets obtained were stained en bloc with 0.5% magnesium uranyl acetate in 0.15 M sodium chloride, then dehydrated, and embedded in Epon in such a way that thin sections could be cut through the entire depth of each pellet. Thin sections were cut with a diamond knife on an MT2 microtome (Ivan Sorvall, Inc., Newtown, Conn.), then stained with uranyl acetate and lead citrate, and examined in a Siemens Elmiskop 101.

**Enzyme Assays**

**GLUCOSE-6-PHOSPHATASE (D-GLUCOSE-6-PHOSPHATE PHOSPHOHYDROLASE; EC 3.1.3.1):** The assay contained 20 mM glucose-6-phosphate and 20 mM sodium acetate buffer, pH 6.6, and from 3.6 to 40 \(\mu\)g fraction protein in 0.5 ml final volume. Activity was measured at 37°C at successive intervals ranging from 0 to 20 min. The reaction was stopped by adding 1.0 ml of 10% TCA, the sample was cleared by centrifugation, and an aliquot of the supernate was assayed for inorganic phosphate by the method of Ames and Dubin (1).

**NADH CYTOCHROME C REDUCTASE (NADH: FERRICYTOTOCHROME B\(_6\) OXIDOREDUCTASE; EC 1.6.2.2):** NADPH CYTOCHROME C REDUCTASE (NADPH: FERRICYTOCHROME OXIDOREDUCTASE; EC 1.6.2.4) AND NADH FERRICYANIDE REDUCTASE (EC 1.6.2.2): The reductases were measured using 1.8-20 \(\mu\)g fraction protein in a 1-ml cuvette with an Acta III Spectrophotometer (Beckman Instruments) by following the reduction of cytochrome at 550 nm or ferricyanide at 420 nm as described by Omura and Takesue (19).

**ACID P-NITROPHENYL PHOSPHATASE (PARAPHOSPHORIC MONOESTER PHOSPHOHYDROLASE; EC 3.1.3.2), ALKALINE PHOSPHODIESTERASE I (PARAPHOSPHORIC DIESTER PHOSPHOHYDROLASE; EC 3.1.4.1):** Acid p-nitrophenyl phosphatase and phosphodiesterase I activities were estimated by measuring the absorbance at 410 nm of p-nitrophenol liberated at 37°C from p-nitrophenylphosphate and p-nitrophenyl thymidine 5'-phosphate respectively, using 28-56 \(\mu\)g fraction protein in a 0.5 ml final volume. The reactions were stopped at intervals ranging from 0 to 20 min by adding 1 ml of glycine buffer, pH 10.7. For acid p-nitrophenyl phosphatase the incubation mixture contained 5 mM sodium acetate buffer pH 5.0, 0.2% Triton X-100, and 5 mM substrate. For alkaline phosphodiesterase I the incubation mixture had 10 mM sodium carbonate/sodium bicarbonate buffer pH 10.0, 2 mM zinc acetate, 0.1% Triton X-100, and 1.5 mM substrate.

**GALACTOSYLTRANSFERASE (UDP-GALACTOSE: ASIALO-GALACTOFETUIN GALACTOSYLTRANSFERASE; EC 2.4.1.38):** The assay measured the transfer of the labeled \([\text{H}]\text{galactose moiety of uridine diposphogalactose (UDP-Gal) to asialo-galactofetuin}, prepared by the method of Kim et al (12). The assay mixture contained (in 0.1 ml final volume) 30 mM sodium cacodylate buffer, pH 6.6, 30
mM manganese chloride, 30 mM 2-mercaptoethanol, 0.2% (wt/vol) Triton X-100, 2 mM adenosine triphosphate, 2 mg asialo-agaractofetuin, 0.39 nM [3H]UDP-Gal and the enzyme suspension (2.8-8.4 μg protein). The assays were carried out at 37°C over intervals ranging from 0 to 20 min and stopped by the addition of 1.0 ml cold 1% phosphotungstic acid in 0.5 N hydrochloric acid. The mixture was centrifuged, and the ensuing pellet was washed twice with 1 ml of the above phosphotungstic acid solution and once with 1.0 ml ethanol at 0°C. The final pellet was resuspended in 1% sodium dodecyl sulfate and counted.

Protein Assay
Protein was determined by the method of Lowry et al (15) using bovine serum albumin as the standard.

RESULTS

Morphological Characterization of $GF_{1+2}$ and RdMF

Two fractions were studied. The first was a combined light Golgi fraction ($G_{F1}$) and intermediate Golgi fraction ($G_{F2}$) designated $G_{F1+2}$, which contains all elements of a total microsomal fraction of density less than 1.11 g/cm³. The morphology of this fraction is shown in Fig. 1, in which a and b illustrate fields at the top and bottom of the pellet, respectively. The fraction consists primarily of morphologically identifiable Golgi elements, that is, vesicles of 200-500 nm in diameter bound by a limiting membrane and

![Figure 1](image-url)
containing dense particles 50-80 nm in diameter, identified as very low density lipoproteins (VLDLs). The top layer of the pellet contains typical Golgi cisternae and, in addition, a significant number of small, empty, smooth-surfaced vesicles of uncertain origin; they could be derived from the smooth ER, Golgi complex, or plasma-lemma. By electron microscope (EM) survey, this fraction has the same degree of homogeneity as the GF₁ and GF₂ (considered together) described in reference 6. The second fraction, RdMF (not illustrated) contains all elements of density greater than 1.15 g/cm³ derived from a total microsomal fraction. It consists primarily of vesicles derived from the smooth and rough ER. Occasionally a morphologically identifiable Golgi cisterna is evident.

Enzyme Activities and their Inactivation

In freshly prepared GF₁+₂ we found significant G-6-Pase and NADPH cyt c reductase activities if we assayed the fractions immediately after their isolation. As seen in Table I the specific activities of these enzymes are ~3 times lower in GF₁+₂ than in the RdMF. These values are most probably underestimates, since they are normalized to total rather than membrane protein. There is considerable variation in the specific activity of the enzymes from one experiment to another, but the relative activity is fairly constant at 30%.

Pertinent data published by Bergeron et al (3) are given for comparison. It should be emphasized that, although the specific activities are higher than previously reported, the G-6-Pase and NADPH cyt c reductase of GF₁+₂ represents a very small proportion of the total cellular activity as shown in Table II. GF₁+₂ contains 12% of the galactosyltransferase, 0.16% of the G-6-Pase, and 0.25% of the NADPH cyt c reductase activities of the whole homogenate. If one accepts the marker enzyme hypothesis (5), these figures indicate that GF₁+₂ is contaminated with elements derived from ER to the extent of 30%, based on both G-6-Pase and NADPH cyt c reductase data.

Both of these enzymes are extensively inactivated when the fractions are suspended in 0.25 M sucrose and stored at 0°C. The inactivation is dependent on storage time and protein concentration as shown in Fig. 2. The NADPH cyt c reductase activity in RdMF is stable at 1.0 and 2.0 mg protein/ml, while at the same protein concentrations that activity in GF₁+₂ is declining.

Above 2 mg protein/ml both enzymatic activities are stable for longer than 24 h in RdMF. It is important to realize that in a typical isolation experiment the protein concentration in RdMF would range from 20 to 40 mg/ml and therefore would have to be diluted significantly before inactivation would occur. In contrast, the protein concentration in GF₁+₂ would typically be be-

### Table I

| Fraction | G-6-Pase | NADPH cyt c reductase |
|----------|----------|-----------------------|
|          | SA* (10) | RAt                  |
| GF₁+₂    | 161 ± 45 | 30 ± 6 [4]            |
| RdMF     | 521 ± 136| 100                   |

The figures in brackets are RA values recalculated from GF₁ + GF₂ and taken directly for total microsomes from Bergeron et al (3); the corresponding SA values were 5 and 130 for G-6-Pase and 8 and 46 for NADPH cyt c reductase. For the latter, the figures were recalculated to give cyt c reduced instead of NADPH oxidized.

* SA = Specific Activity = nmol/min/mg protein ± s.d. Number of experiments is shown in parentheses.
† RA = Relative Activity of GF₁+₂ given as percent of RdMF activity.

### Table II

| Protein and Enzyme Activity Recovered in Golgi and Microsomal Fractions |
|-----------------------------|----------|-----------------------------|
|                             | GF₁+₂ (4) | RdMF (4)                    |
| Proline                     | 0.22* ± 0.08 | 14.02* ± 0.44              |
| Glucose-6-Phosphatase       | 0.16 ± 0.07  | 47.10 ± 2.49               |
| NADPH cyt c reductase       | 0.25 ± 0.08  | 47.76 ± 1.66               |
| Galactosyltransferase       | 12.47 ± 3.36 | 21.50 ± 5.60               |

Number of experiments given in parentheses.

* Percent of whole homogenate.
FIGURE 2 Loss of NADPH cyt c reductase and G-6-Pase activities in GF<sub>1+2</sub> and RdMF with time. Protein concentrations in mg/ml are given directly on the graphs. Left panels: NADPH cyt c reductase activity: 100% Activity GF<sub>1+2</sub> = 39 nmol/min/mg and in RdMF = 126 nmol/min/mg. Relative activity (RA) of GF<sub>1+2</sub> with respect to RdMF = 31%. Right panels: G-6-Pase activity: 100% Activity GF<sub>1+2</sub> = 174 nmol/min/mg and in RdMF = 635 nmol/min/mg. RA of GF<sub>1+2</sub> with respect to RdMF = 27%. The fractions were removed from the gradient and their absorption at 280 nm was measured. The protein concentration was then calculated from standard curves correlating for each fraction absorbance at 280 nm with protein content determined by the Lowry et al. procedure (15). The fractions were concentrated or diluted to the appropriate protein concentration and to a final sucrose concentration 0.25 M, then stored at 0°C and assayed at the designated time points.

Prevention of the Inactivation

Of the many substances that we have tested (proteolytic inhibitors-soybean trypsin inhibitor at 1 mg/ml, leupeptin from 0.1 to 10.0 μg/ml, and pepstatin from 0.01 to 1.0 μg/ml (2); antioxidants...
EDTA and catalase have been found to be most effective at low concentrations in preventing the specific inactivation of both G-6-Pase and NADPH cyt c reductase (Fig. 3). Higher concentrations of both EDTA and catalase are required for the protection of G-6-Pase activity than for that of NADPH cyt c reductase activity.

Since it has already been demonstrated (Fig. 2) that inactivation is slower in more concentrated fractions, it was necessary to determine if the effect of catalase was specific. Catalase was compared with two other proteins, bovine serum albumin and superoxide dismutase as shown in Fig. 4. In each case catalase was the most effective protectant for G-6-Pase activity. Boiled catalase, with no assayable enzymatic activity, at 32 μM, was 50% as effective as enzymatically active catalase in protecting the activities mentioned. Therefore, catalase appears to protect by two different mechanisms: (a) by increasing the protein concentration, and (b) by its specific enzymatic activity.

Morphology of GF1+2 and RdMF after Storage

Significant morphological changes occur in the fractions when stored at 0°C for 18 h at low protein concentration (0.25-0.40 mg/ml) i.e., under conditions leading to maximal inactivation. In GF1+2 (Fig. 5) there is extensive membrane damage leading to vesicle breakage and liberation of VLDLs (Fig. 5b) with concomitant VLDL fusion into lipid droplets as large as 1.5 μm, both inside (Fig. 5b) and outside Golgi vesicles (Fig. 5c). The freed VLDLs stick to membranes (Fig. 5a, b, and c) and occasionally form mixed aggregates (Fig. 5a). Besides vesicle breakage, membrane damage includes reorganization into large sheets with patchy discontinuities (Fig. 5c) and fragmentation which generates free membrane edges.

The RdMF undergoes much less drastic changes than does the Golgi fraction over 18 h storage at 0°C. As seen in Fig. 6, the vesicles remain about the same size. Although there is an increase in irregular vesicle profiles and an increased inci-

![Figure 3](image)

**Figure 3** NADPH cyt c reductase and G-6-Pase activities in GF1+2 and RdMF. Protection against inactivation with EDTA and catalase. GF1+2 and RdMF were both suspended in 0.25 M sucrose at 0.24 mg protein/ml and stored at 0°C. Activity was determined after 18 h storage in the presence of EDTA or catalase and is expressed as percent of activity at t = 0. NADPH cyt c reductase activity (100% Activity in GF1+2 = 28 nmol/min/mg and in RdMF = 88 nmol/min/mg). RA of GF1+2 with respect to RdMF = 32%. Left column: G-6-Pase activity (100% Activity in GF1+2 = 97 nmol/min/mg and in RdMF = 459 nmol/min/mg). RA of GF1+2 with respect to RdMF = 21%. In separate experiments, NADPH cyt c reductase was protected to the extent of 60-70% at lower EDTA concentrations, e.g., 0.01 mM for RdMF and 0.025 mM for GF1+2.

![Figure 4](image)

**Figure 4** NADPH cyt c reductase and G-6-Pase activities in GF1+2 and RdMF. Protection against inactivation with catalase, bovine serum albumin and superoxide dismutase. GF1+2 and RdMF were both suspended in 0.25 M sucrose at 0.18 mg protein/ml and stored at 0°C. Activity was determined after 18 h storage in the presence of catalase (O—O), bovine serum albumin (B—B), and superoxide dismutase (D—D). Left column: NADPH cyt c reductase activity: 100% activity in GF1+2 = 43 nmol/min/mg and in RdMF = 150 nmol/min/mg. RA of GF1+2 with respect to RdMF = 28%. Right column: G-6-Pase activity: 100% activity in GF1+2 = 164 nmol/min/mg and in RdMF = 567 nmol/min/mg. RA of GF1+2 with respect to RdMF = 29%.
FIGURE 5  $G_{F_{1+2}}$ stored at a protein concentration of 0.40 mg/ml, after 18 h at 0°C, sectioned pellets.  
(a) Field from the top of the pellet showing swollen vesicles still containing a few VLDLs ($sL_1$), freed VLDLs ($p$), and VLDLs fused to vesicles ($sL_2$). (b) Field from the middle of the pellet where breakage of Golgi vesicles ($gv$) is evident (arrows) and VLDL fusion leads to formation of large lipid droplets ($fb$). (c) Field from the bottom of the pellet where membrane fragmentation generates free membrane edges ($fe$) damaged membrane is reorganized into large sheets ($ms$) and VLDLs fuse into large lipid droplets ($fp$). $x$ 29,500

dence of membrane invaginations (vesicles within vesicles) and of ribosomal “capping,” there is no indication of gross lipid rearrangement or severe membrane damage of the type seen in $G_{F_{1+2}}$.

Effect of Ethanol Pretreatment

All the data presented were obtained in experiments in which rats were pretreated with ethanol to enhance the yield of $G_{F_{1+2}}$. This treatment did not alter the level of activity of G-6-Pase or NADPH cyt c reductase in the recovered $G_{F_{1+2}}$. The inactivation profile and the morphological changes were identical in control and ethanol treated rats.

DISCUSSION

We have detected relatively high specific activities of G-6-Pase and NADPH cyt c reductase—two enzymes thought to be restricted to ER membranes—in isolated Golgi fractions assayed immediately after their isolation. Data obtained (but not presented) indicate that this activity does not represent an increase in the contamination of the fractions by ER elements ascribable to variations in animal sources or poorly controlled variables in the homogenization-cell fractionation procedures. Since the enzymes are specifically inactivated by storage at 0°C in 0.25 M sucrose, and since (for reasons of convenience) assays are often carried out on stored fractions, we assume that inactivation explains the low specific activities of G-6-Pase and NADPH cyt c reductase reported in Golgi fraction by Bergeron et al (3), it may also have affected data reported by other investigators using different isolation procedures (8, 17).

Histochemical tests for G-6-Pase on either liver or hepatic cell fractions are reported as negative in Golgi elements (22, 14, 7). The apparent discrepancy between these tests and our data could again be explained by the lability of the Golgi G-6-Pase activity.

The reactions involved in the inactivation of these two membrane proteins are unknown at

2 This was the case in the experiments previously carried out in our laboratory and reported by Bergeron, Ehrenreich, Siekevitz, and Palade (3).

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phospholipid and triacylglycerol synthesis [23] proven to be restricted to the ER. The usefulness of morphology is only limited because in all Golgi fractions there are a few small, empty vesicles of possible ER origin. To answer this question, we have taken an immunological approach which is described in the companion paper (9). Since NADPH cyt c reductase has been isolated and antibodies have been made against the isolated enzyme (19, 13), it should be possible to use this antibody (immobilized to a convenient substrate) to determine the morphological identity of the constituents of GF1+2 which react with the anti-NADPH cyt c reductase.

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