

ISOLATION OF A GOLGI APPARATUS-RICH FRACTION FROM RAT LIVER

IV. Thiamine Pyrophosphatase

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ABSTRACT

The thiamine pyrophosphatase (the enzyme [s] catalyzing the release of inorganic phosphate with thiamine pyrophosphate as the substrate) activities of Golgi apparatus-, plasma membrane-, endoplasmic reticulum-, and mitochondria-rich fractions from rat liver were compared at pH 8. Activity was concentrated in the Golgi apparatus fractions, which, on a protein basis, had a specific activity six to eight times that of the total homogenates or purified endoplasmic reticulum fractions. However, only 1–3% of the total activity was recovered in the Golgi apparatus fractions under conditions where 30–50% of the UDPgalactose:N-acetylglucosamine-galactosyl transferase activity was recovered. Considering both recovery of galactosyl transferase and fraction purity, we estimate that approximately 10% of the total thiamine pyrophosphatase activity of the liver was localized within the Golgi apparatus, with a specific activity of about ten times that of the total homogenate. Cytochemically, reaction product was found in the cisternae of the endoplasmic reticulum as well as in the Golgi apparatus. This is in contrast to results obtained in most other tissues, where reaction product was restricted to the Golgi apparatus. Thus, enzymes of rat liver catalyzing the hydrolysis of thiamine pyrophosphate, although concentrated in the Golgi apparatus, are widely distributed among other cell components in this tissue.

INTRODUCTION

In many tissues and cell types, thiamine pyrophosphatase (TPPase) has been assumed (3, 4, 10, 11, 16, 17, 22) or suggested (1, 18, 20) to be a marker enzyme specific for the Golgi apparatus. Other studies indicate that TPPase may be a marker enzyme for the Golgi apparatus of some tissues but not of others (7, 8, 10). These reports have dealt with the enzyme by light or electron microscope cytochemistry. Here we compare purified fractions of endoplasmic reticulum, Golgi apparatus, plasma membrane, and mitochondria

isolated from rat liver to determine if TPPase is a marker specific for the Golgi apparatus of this tissue, and to correlate these biochemical findings with those obtained cytochemically.

MATERIALS AND METHODS

Cell Fractions

Golgi apparatus-, endoplasmic reticulum-, plasma membrane-, and mitochondria-rich fractions were prepared as described previously (5, 14, 15, Fig. 1)

from 50 day-old rats (Holtzman Company, Madison, Wisconsin) fed Purina Laboratory Chow. Enzyme assays were performed at 37°C under conditions where activity was proportional to the time of incubation (Fig. 2 a) and protein concentration (Fig. 2 b). The assay medium was a modification of that of Allen and Slater (3) and contained 33 mM sodium barbital, 15 mM calcium chloride, and 5 mM sodium β -glycerol phosphate (Sigma Chemical Co., St. Louis, Mo.) or 3.3 mM thiamine pyrophosphate (Sigma). The assay was initiated by adding 0.1 ml of resuspended fraction or total homogenate to 1.9 ml of medium, and the reaction was terminated by adding 1.0 ml of cold 15% trichloroacetic acid. Protein was determined by the method of Lowry et al. (13), and inorganic phosphate by the method of Berenblum and Chain as modified by Martin and Doty (see ref. 12). Data were corrected for nonspecific hydrolysis by subtracting appropriate substrate and enzyme

controls. UDPgalactose:*N*-acetylglucosamine-galactosyl transferase (galactosyl transferase) was assayed as previously described (14).

Cytochemistry

Tissue was obtained from 10-week-old male Sprague-Dawley rats. Small blocks of liver, pancreas, and epididymis were fixed in cold 1% paraformaldehyde and 3% distilled glutaraldehyde in 0.067 M sodium cacodylate buffer, pH 7.4. Nonfrozen sections were prepared on a Smith-Farquhar TC-2 tissue sectioner and incubated for 1–1.5 hr at pH 7.4 in the medium employed by Novikoff and Goldfischer (17). Controls were incubated at pH 5, with 0.1 M NaF added to the medium (pH 7.4), or without the substrate (pH 7.4).

After incubation, the tissue was postfixed for 1.5 hr at room temperature in acetate-Veronal-buffered 1% OsO₄ (pH 7.4) with 5% sucrose, treated with buffered 0.5 M uranyl acetate for 1 hr at room temperature, dehydrated in graded ethanols, and embedded in Epon. Thin sections were prepared with a Sorvall MT-2 microtome (Ivan Sorvall, Inc., Norwalk, Conn.) equipped with a diamond knife, and stained with alkaline lead. Electron micrographs were taken with a Siemens Elmiskop Ia operating at 80 kv, with a double condenser and a 50 μ molybdenum objective aperture.

RESULTS

Cell Fractions

The pH curve for TPPase of purified Golgi apparatus indicates an optimum near pH 8 and a shoulder of activity in the alkaline region (Fig. 3 a). Traces of alkaline phosphatase remaining in the Golgi apparatus-rich fraction may con-

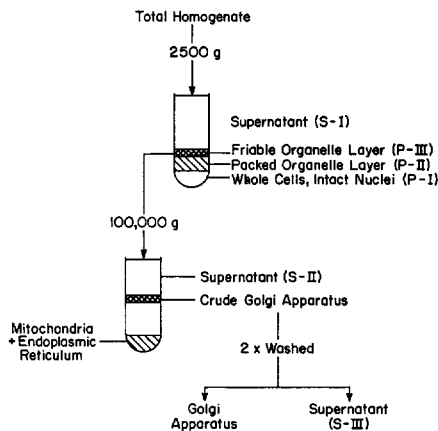


FIGURE 1 Procedure for isolation of Golgi apparatus. For details, see Morr e et al. (14, 15).

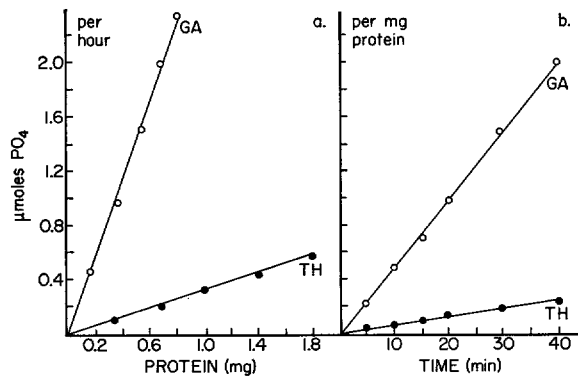


FIGURE 2 Thiamine pyrophosphatase activity in a Golgi apparatus-rich fraction. GA, Golgi apparatus; TH, total homogenate. TPPase activity was determined from the release of inorganic phosphate. All assays were performed at 37°C, pH 8.0.

tribute to the shoulder. The pH optimum for the alkaline phosphatase activity of an unwashed Golgi apparatus fraction is approximately 9 (Fig. 3 c). Washing the Golgi apparatus-rich fraction produces a 2- to 3-fold reduction in this activity (Fig. 3 d), whereas with TPPase, the Golgi apparatus-rich fraction increases in specific activity during washing (Fig. 3 b). TPPase activity of other fractions is reduced to the level of the total homogenate, or lower, by washing. The twice-washed Golgi apparatus-rich fractions had a specific activity ($3.2 \pm .05 \mu\text{moles PO}_4/\text{hr per mg protein}$) as much as eight times that of the total homogenate (Fig. 2), but only about 0.5% of the total TPPase activity was recovered in this fraction (Table I). Because of this low recovery, the distribution of TPPase was compared with that of galactosyl transferase, an established marker enzyme for rat liver Golgi apparatus (9, 14, 19, 21). In these experiments, 5 mM mercaptoethanol was added to the homogenization medium, since sulfhydryl protectants are needed

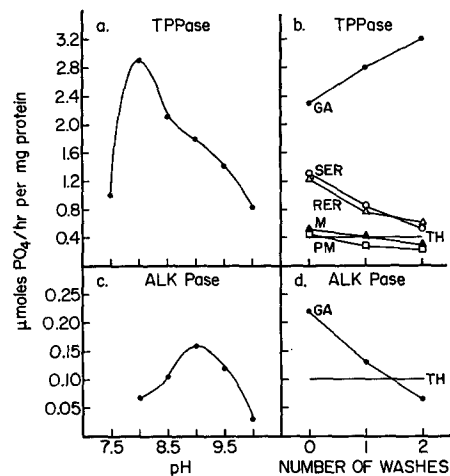


FIGURE 3 The effect of pH and washing on the distribution of thiamine pyrophosphatase and alkaline phosphatase activity in Golgi apparatus-rich fractions. *ALK Pase*, alkaline phosphatase; *TPPase*, thiamine pyrophosphatase; *GA*, Golgi apparatus; *SER*, smooth-endoplasmic reticulum; *RER*, rough-surfaced endoplasmic reticulum; *M*, mitochondria; *PM*, plasma membrane; *TH*, total homogenate. The pH was adjusted just before assay by the addition of HCl or NaOH. Activity was determined from the release of inorganic phosphate at 37°C. In Figs. 3 b and 3 d the total homogenate was not washed and its specific activity is shown as a horizontal reference line.

TABLE I
Distribution of Thiamine Pyrophosphatase Activity during Rat Liver Cell Fractionation

| Fraction | Specific activity* | Total activity† | Recovery % |
|-------------------|--------------------|-----------------|------------|
| Total homogenate | 0.49 | 198.7 | — |
| P-I + P-II | 0.34 | 53.4 | |
| S-I | 0.60 | 64.7 | |
| P-III | 0.77 | 41.0 | |
| | | 159.1 | 80.1 |
| S-II | 0.31 | 3.2 | |
| Golgi Apparatus | 3.03 | 1.0 | |
| Mitochondria + ER | 0.69 | 25.2 | |
| S-III | 0.67 | 1.8 | |
| | | 31.2 | 75.9 |

Yield of TPPase activity in the Golgi apparatus fraction is $1/198.7 = 0.5\%$.

* Units of specific activity are $\mu\text{moles inorganic phosphate per hr per mg protein}$.

† Total activity was determined by multiplying the $\mu\text{moles phosphate released per ml}$ by the total fraction volume in ml.

For isolation procedure see Fig. 1. P-I, whole cells and intact nuclei, P-II, packed organelle layer; P-III, friable organelle layer; S-I, low speed supernatant; S-II, high speed supernatant; S-III, Golgi apparatus washes; ER, endoplasmic reticulum.

to maintain the activity of galactosyl transferases (14, 21). Mercaptoethanol doubled the TPPase activity of the Golgi apparatus-rich fraction and increased the activity of the total homogenate 1.5–2-fold. Golgi apparatus were recovered from all layers of the 2000 g pellet above the layers of intact cells and unbroken nuclei (Fig. 1). When the recoveries of TPPase and galactosyl transferase were followed simultaneously under these conditions, about 40% of the galactosyl transferase activity was recovered in the purified Golgi apparatus fraction, in contrast to 1.6% of the TPPase activity.

Cytochemistry

In rat liver incubated for TPPase, lead phosphate reaction product is frequently present in the smooth- and rough-surfaced endoplasmic

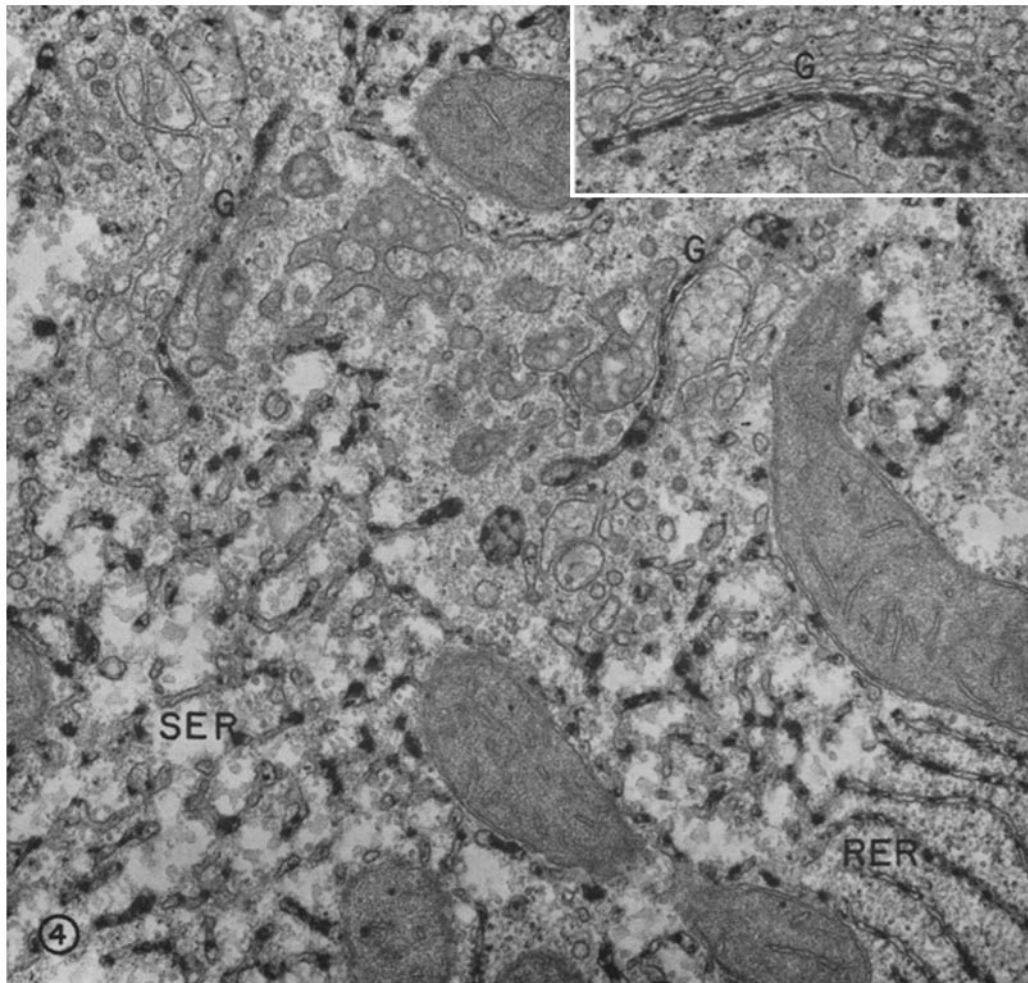
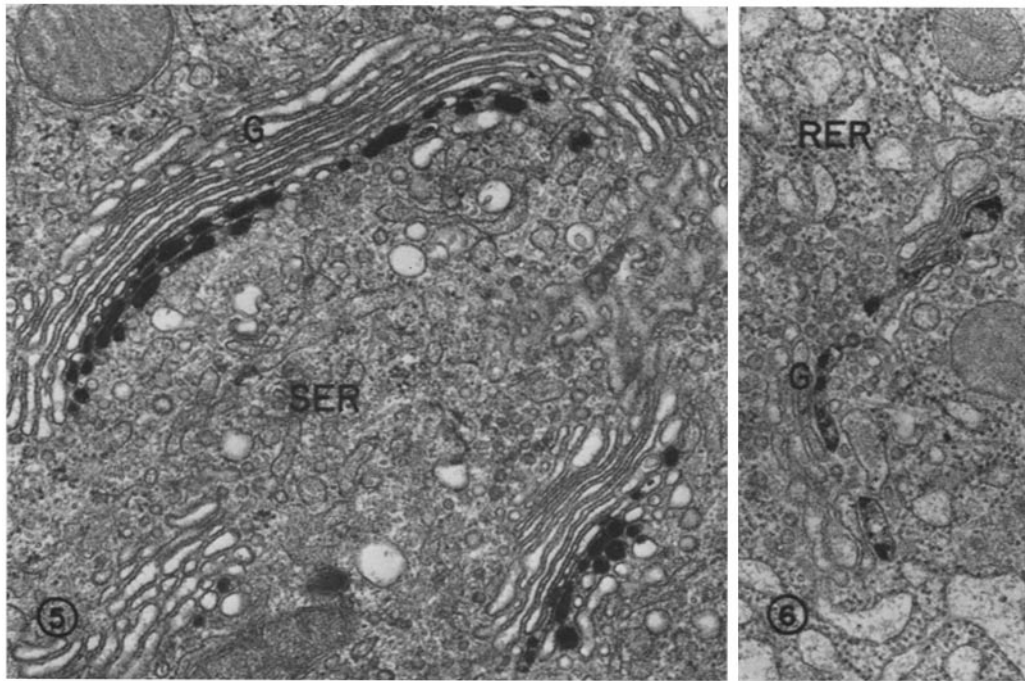


FIGURE 4 Electron micrograph of rat liver incubated for TPPase activity. Lead phosphate reaction product is present in Golgi apparatus cisternae (*G*), smooth-surfaced endoplasmic reticulum (*SER*), and rough-surfaced endoplasmic reticulum (*RER*). *Inset*: In sections perpendicular to the long axis of the Golgi apparatus, reaction product is seen within two cisternae along the inner, concave surface of the Golgi apparatus. While this distribution and concentration of reaction product within the Golgi apparatus are similar to those seen in other tissues (compare inset with Figs. 5 and 6), the large amount of reaction product within the endoplasmic reticulum (Fig. 4) limits its use as a specific marker for the Golgi apparatus in rat liver. $\times 52,000$; inset $\times 50,000$.

reticulum as well as in one or two cisternae of the Golgi apparatus (Fig. 4). In sections perpendicular to the long axis of a stack of curved Golgi cisternae (Fig. 4, inset), reaction products are seen in the inner, concave cisternae, which are similar to those in the Golgi apparatus of epididymis (Fig. 5) and pancreas (Fig. 6). Reaction product, however, is absent from the endoplasmic reticulum in the epididymis and pancreas.

DISCUSSION

A true marker enzyme for a specific cell fraction, e.g., succinic dehydrogenase for mitochondria, fulfills the following criteria: (a) The specific activity of the enzyme is several times greater than that of the total homogenate, (b) the specific activity of the enzyme increases upon purification of the membrane fraction, (c) the activity



FIGURES 5 and 6 Golgi regions of rat epididymal epithelial cell (Fig. 5) and pancreatic acinar cell (Fig. 6) prepared for the demonstration of TPPase in the same manner as the liver cell in Fig. 4. Notice the specific localization of reaction product within the inner, concave cisternae of the Golgi apparatus (*G*) and the absence of reaction product in regions of smooth- (*SER*) and rough- (*RER*) surfaced endoplasmic reticulum in these cell types. Fig. 5, $\times 32,000$; Fig. 6, $\times 36,000$.

is low or absent in other purified cell fractions, and (*d*) the fraction containing the enzyme comprises a percentage of the total activity commensurate with the percent recovery of the cell component involved.

TPPase fulfills the first two criteria as a Golgi apparatus marker in rat liver (Fig. 3). The relative specific activity of an unwashed Golgi apparatus-rich fraction is five times that of the total homogenate and increases to six-eight times that of the total homogenate in twice-washed preparations. However, the increase in the relative specific activity of the Golgi apparatus fraction after washing may be related to enzyme activation (23), accessibility to substrate, or loss of contaminating membrane components. Our results (see also refs. 6 and 19) contrast with those of Fleischer et al. (9) who report no concentration of TPPase in their Golgi apparatus fractions isolated by zonal centrifugation from bovine liver and assayed by the procedures of Yamazaki and Hayashi (23).

Enzymes catalyzing the hydrolysis of thiamine

pyrophosphate do not fulfill the last two criteria as specific markers for rat liver Golgi apparatus. By biochemical assay, rough- and smooth-endoplasmic reticulum, mitochondria, and plasma membrane reveal such activity. Although the specific activity of these membranes decreases with washing, some activity remains in the twice-washed fractions. Recovery experiments (Table II) demonstrate that the Golgi apparatus contains only 1-3% of the total TPPase activity of the homogenate, in contrast to 30-50% recovery of galactosyl transferase in the same experiments. Galactosyl transferase may be localized in the Golgi apparatus of rat liver (14, 21) and other tissues (9, 19). Recoveries as high as 70% have been reported (14), with an average of 40-50% (14, 21). These data indicate that other components of the endomembrane system, as well as plasma membrane and mitochondria, contain most of the TPPase activity. While the specific activity of these fractions is low, they represent a large percentage of the total membrane protein and contain most of the activity. Moreover, previ-

TABLE II
Comparison of the Recovery of Thiamine Pyrophosphatase and Galactosyl Transferase in the Golgi Apparatus-Rich Fraction

| Fraction | Enzyme | Specific activity* | | | | Mean \pm average deviation | RSA† |
|--|-----------|--------------------|-------|-------|-------|------------------------------|------|
| | | 1 | 2 | 3 | 4 | | |
| Total homogenate | TPPase | 1.11 | 0.92 | 0.84 | 0.98 | 0.96 \pm 0.08 | — |
| | Gal. Tase | 2.8 | 1.6 | 2.6 | 2.8 | 2.5 \pm 0.40 | — |
| Golgi apparatus | TPPase | 4.06 | 3.88 | 4.25 | 3.75 | 3.98 \pm 0.17 | 4.1 |
| | Gal. Tase | 248.0 | 214.0 | 182.0 | 191.0 | 209.0 \pm 22.2 | 85.0 |
| % of activity in total homogenate recovered in Golgi apparatus | | | | | | | |
| | TPPase | 1.1 | 1.6 | 1.0 | 2.8 | 1.6 \pm 0.6 | — |
| | Gal. Tase | 31.9 | 48.6 | 28.2 | 48.4 | 39.2 \pm 9.2 | — |

* TPPase assayed at pH 8.0 as described in the text, with specific activity expressed as μ moles phosphate per hr per mg protein. UDP Gal. Tase assayed as described in the text, with specific activity expressed as μ moles *N*-acetylglucosamine-dependent UDP-galactose hydrolysis per hr per mg protein. Results are from unwashed Golgi apparatus fractions.

† Relative specific activity equals the ratio of specific activity of the fraction to that of the total homogenate.

ous histochemical studies, in addition to the cytochemical data presented in this paper, have demonstrated TPPase in endoplasmic reticulum of rat liver (8, 10) and other tissues (8, 16).

Two observations indicate that the TPPase activity in the Golgi apparatus-rich fraction is not due to residual alkaline phosphatase. The pH optimum for alkaline phosphatase is 9.0, whereas that for TPPase is 8.0. Washing the Golgi apparatus-rich fraction reduces the alkaline phosphatase activity as it increases the TPPase activity. A significant portion of the TPPase activity exhibited by the plasma membrane fractions and the total homogenate might be logically attributed to alkaline phosphatase. Conversely, the endogenous alkaline phosphatase activity of endoplasmic reticulum, the major source of membrane protein in rat liver homogenates, is too low to account for the rates of thiamine pyrophosphate hydrolysis by endoplasmic reticulum fractions in this study.

Liver microsomes contain an active nucleoside diphosphatase (pH 7.0) specific for inosine diphosphate (IDP), uridine diphosphate (UDP), and guanosine diphosphate (GDP) (adenosine diphosphate [ADP] is not hydrolyzed and cytidine diphosphate [CDP] is hydrolyzed more slowly) (23) (on a specific activity basis, endoplasmic reticulum > Golgi apparatus > plasma membrane [plasma membrane > Golgi apparatus > endoplasmic reticulum for ADP and CDP] [5]). This enzyme

also catalyzes the hydrolysis of thiamine pyrophosphate with a pH optimum of about 8.8 (23) and could account for the TPPase activity of the endoplasmic reticulum fractions in liver. Thus, we do not exclude the possibility that the TPPase localized in the liver Golgi apparatus is a unique enzyme. This has been demonstrated by Allen (2) in epididymis where the Golgi apparatus enzyme accounts for a greater proportion of the total activity (1, Fig. 5).

In any event, the results show that the substrate thiamine pyrophosphate, at the pH optimum for thiamine pyrophosphate hydrolysis by the Golgi apparatus, cannot be used as a biochemical marker for enzymes of the Golgi apparatus of rat liver. Despite the failure of TPPase to meet the rigorous definition for a marker enzyme in rat liver, TPPase is of value as a cytochemical marker for the Golgi apparatus of other cell types, such as in the epididymis and pancreas, in view of its high specific activity relative to other cell components. In contrast to TPPase, galactosyl transferase appears to fulfill all criteria as a true marker for the Golgi apparatus of rat liver.

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