ISOLATION OF A GOLGI APPARATUS-RICH FRACTION FROM RAT LIVER

II. Enzymatic Characterization and Comparison with Other Cell Fractions

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ABSTRACT

Enzymatic activities associated with Golgi apparatus-, endoplasmic reticulum-, plasma membrane-, mitochondria-, and microbody-rich cell fractions isolated from rat liver were determined and used as a basis for estimating fraction purity. Succinic dehydrogenase and cytochrome oxidase (mitochondria) activities were low in the Golgi apparatus-rich fraction. On the basis of glucose-6-phosphatase (endoplasmic reticulum) and 5′-nucleotidase (plasma membrane) activities, the Golgi apparatus-rich fraction obtained directly from sucrose gradients was estimated to contain no more than 10% endoplasmic reticulum- and 11% plasma membrane-derived material. Total protein contribution of endoplasmic reticulum, mitochondria, plasma membrane, microbodies (uric acid oxidase), and lysosomes (acid phosphatase) to the Golgi apparatus-rich fraction was estimated to be no more than 20–30% and decreased to less than 10% with further washing. The results show that purified Golgi apparatus fractions isolated routinely may exceed 80% Golgi apparatus-derived material. Nucleoside di- and triphosphatase activities were enriched 2–3-fold in the Golgi apparatus fraction relative to the total homogenate, and of a total of more than 25 enzyme-substrate combinations reported, only thiamine pyrophosphatase showed a significantly greater enrichment.

INTRODUCTION

The identification of Golgi apparatus isolated from rat liver was established in the preceding paper (17) by electron microscopy of thin sections of pellets and negatively stained preparations. In this report, we have utilized the technique for Golgi apparatus isolation to search for specific enzymatic characteristics of this cell component and to distinguish it from other cell fractions. For comparative purposes, endoplasmic reticulum-, plasma membrane-, mitochondria-, and microbody-rich fractions were prepared and assayed for enzymatic activities characteristic of single cell components. These results were then used to provide a quantitative estimate of the relative purity of the Golgi apparatus fraction on a protein basis.

MATERIALS AND METHODS

Golgi apparatus-rich fractions were prepared as described in the preceding report (17). The plasma membrane fraction was obtained by a modification (10) of Neville's (21) procedure. Microbodies were isolated by sucrose gradient centrifugation (7, 8). Mitochondria were recovered from the 1.6–1.8 M interface of the sucrose gradients used to obtain Golgi apparatus (17) and were purified through two

centrifugation-resuspension cycles (30 min, 9000 *g*, 8,500 rpm, Servall HS). All preparative steps were conducted at 0–4°.

Endoplasmic reticulum was obtained from homogenates of rat liver prepared in a manner identical to those used for Golgi apparatus isolation (17). The homogenate was then centrifuged for 10 min at 15,000 *g* (12,500 rpm, Spinco 39 SW) and the supernatant was layered on a discontinuous sucrose gradient consisting of 2.0 M sucrose and 1.5 M sucrose in a v/v ratio to sample of 0.5:0.5 (5). Gradients were centrifuged for 4.5 hr at 100,000 *g* (33,000 rpm, Spinco 39 SW). The sedimented component distributed into two bands at the 1.5–2.0 M sucrose interface. The lower band containing predominantly vesicles of rough-surfaced endoplasmic reticulum was removed from the gradient and resuspended in deionized water.

All enzyme assays were done at 37° under conditions in which activity was verified to be proportional to time of incubation and protein concentration. The following enzymatic activities were determined according to the procedures referenced: glucose-6-phosphatase, EC 3.1.3.9 (26); succinic dehydrogenase (succinate dehydrogenase), EC 1.3.99.1 as succinate-2-(*p*-indophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium (INT) reductase (22); 5′-nucleotidase (5′-mononucleotidase), EC 3.1.3.5 (10); cytochrome oxidase, EC 1.9.3.1 (25); uric acid oxidase (urate: O_2-oxidoreductase), EC 1.7.3.3 (14). Protein was determined by the Lowry procedure (15). Inorganic phosphate was determined by the method of Fiske and Subbarow (12).

Substrates were of the highest purity obtainable from the suppliers indicated: cytochrome *c*, inosine-5′-monophosphate (IMP), inosine-5′-diphosphate (IDP), uridine-5′-monophosphate (UMP), thymidine-5′-monophosphate (TMP), thymidine-5′-diphosphate (TDP), thymidine-5′-triphosphate (TTP) and guanosine-5′-diphosphate (GDP) (Calbiochem, Los Angeles); uric acid, thiamine pyrophosphate (TPP), adenosine-5′-diphosphate (ADP), cytidine-5′-diphosphate (CDP), cytidine-5′-triphosphate (CTP), uridine-5′-diphosphate (UDP), inosine-5′-triphosphate (ITP), uridine-5′-triphosphate (UTP) (Sigma Chemical Co., St. Louis); glucose-6-phosphate, adenosine-5′-monophosphate (AMP), and guanosine-5′-monophosphate (GMP) (Mann Research Laboratories, New York); and INT, adenosine-5′-triphosphate (ATP) and cytidine-5′-monophosphate (CMP) (Nutritional Biochemical Corp., Cleveland).

RESULTS

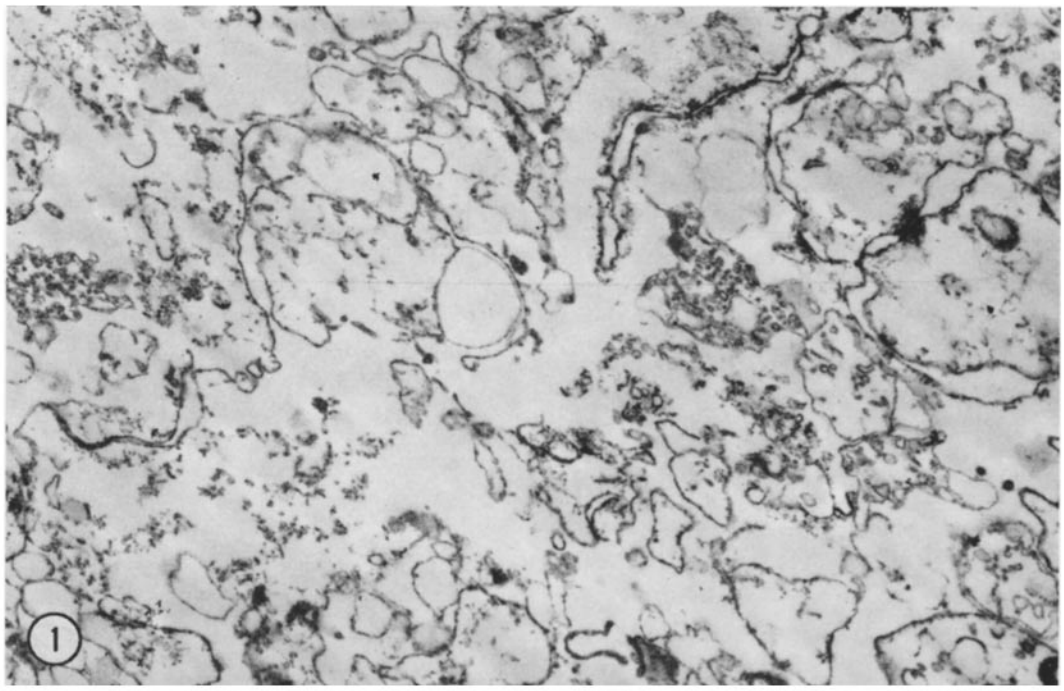
Plasma Membrane–Rich Fraction

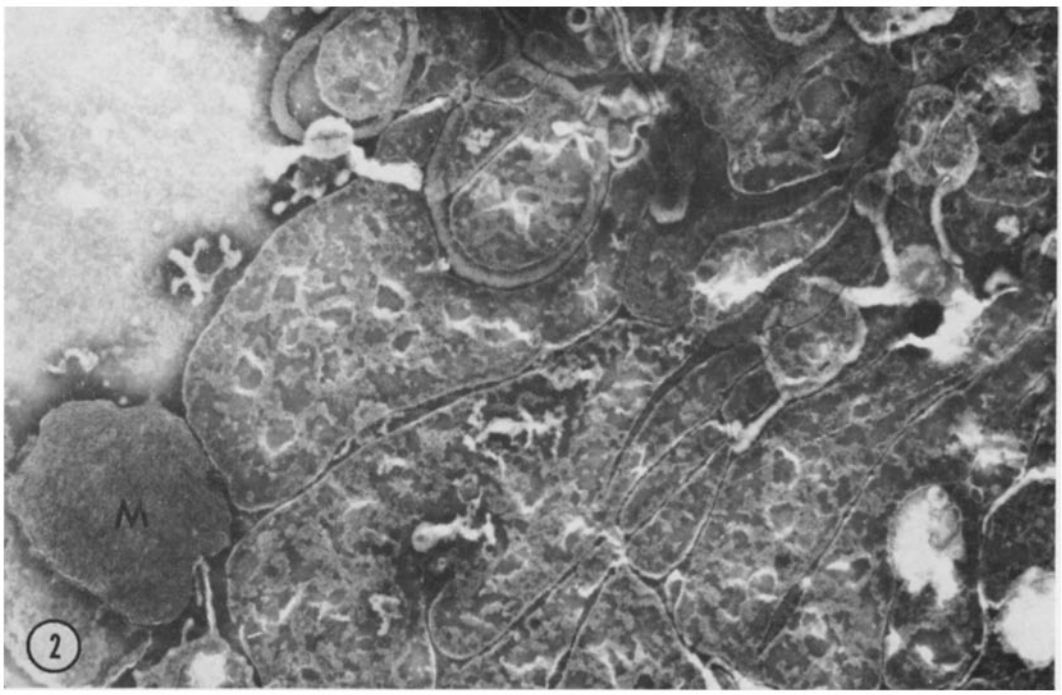
The yield of plasma membrane was 3–4 mg of protein from 10 g fresh weight of liver. Preparations contained vesicles of many sizes and occasionally desmosomes, both of which are features of plasma membrane fractions (Fig. 1). After staining with phosphotungstate (PTA), most of the collapsed membranes showed a fine, granular structure with smooth edges in surface view (Fig. 2). Membrane margins sometimes exhibited globular knobs or the hexagonal array of subunits as reported by Benedetti and Emmelot (2–4; Figs. 2 and 3 of reference 16). The plasma membrane fraction from livers of the Holtzman strain of rats was difficult to free of mitochondrial contamination, a difficulty not encountered with livers from other rat strains or with bovine mammary tissue (Keenan et al., in preparation). Based on estimates of succinic-INT-reductase, the specific activity of the plasma membrane fraction was about 12% that of our washed mitochondria (Tables I, IV) and about 8% that of a purified mitochondrial fraction (23). The relative specific activity of glucose-6-phosphatase was about 12% that of purified endoplasmic reticulum (Tables II, IV). The plasma membrane fractions exhibited at least a 30-fold enrichment of Na^+-activated ATPase (16) and a 25-fold enrichment of 5′-nucleotidase as compared to the total homogenate (Table I).

Endoplasmic Reticulum–Rich Fraction

The yield of endoplasmic reticulum was 5–10 mg of protein from 10 g fresh weight of liver, but on the basis of estimates of glucose-6-phosphatase activity this preparation appeared to represent a relatively small percentage of the total endoplasmic reticulum of homogenates. The procedure involved an uncomplicated discontinuous gradient (5) which selected on the basis of density for membranes with ribosomes attached. Although yields were low, fraction purity was high (Fig. 3, Table II). Measurements of succinic-INT-reductase show less than 2% contamination of this fraction by mitochondria or mitochondrial fragments on a protein basis (Tables II, IV). The plasma membrane contribution to the endoplasmic reticulum–rich fraction based on estimates of 5′-nucleotidase activity was less than 0.5% of the total protein. UDP-N-acetylglucosamine transferase activity (28, 29) could not be demonstrated. Glucose-6-phosphatase was enriched 9-fold relative to the total homogenate. Recovery experiments showed that the loss of glucose-6-phosphatase activity during centrifugation was negligible.

Endoplasmic reticulum preparations contained





FIGURES 1 and 2 Plasma membrane–rich fraction from rat liver in thin section (Fig. 1, × 17,000) and negative contrast (Fig. 2, × 28,000). *M*, mitochondrion.

TABLE I

Enzymatic Activity of Plasma Membrane-Rich Cell Fraction

Enzyme	Specific activity*			Relative specific activity‡
	1	2	3	
5′-Nucleotidase (AMP)	42.2	43.5	40.0	41.9 ± 1.2
Glucose-6-phosphatase	1.8	1.2	1.2	1.1 ± 0.1
Succinate-INT-reductase	1.9	2.6	1.3	1.2 ± 0.5

* Units of specific activity are μmoles of inorganic phosphate/hr per mg of protein (using assay conditions reported by Emmelot et al. [10]) except for succinate-INT-reductase, which is given as μmoles INT reduced/hr per mg of protein (22). Values in each determination are from different animals and not directly comparable.

‡ Ratio of specific activity of plasma membrane–rich fraction to that of total homogenate ± average deviation.

TABLE II

Enzymatic Activities of the Endoplasmic Reticulum-Rich Fraction

Enzyme	Specific activity*			Relative specific activity‡
	1	2	3	
Glucose-6-phosphatase	12.8	10.4	10.9	8.8 ± 1.0
5′-Nucleotidase (AMP)	0.1	0.1	0.2	0.1 ± 0.0
Succinate-INT-reductase	0.2	0.2	0.3	0.2 ± 0.04

* Units as in Table I.

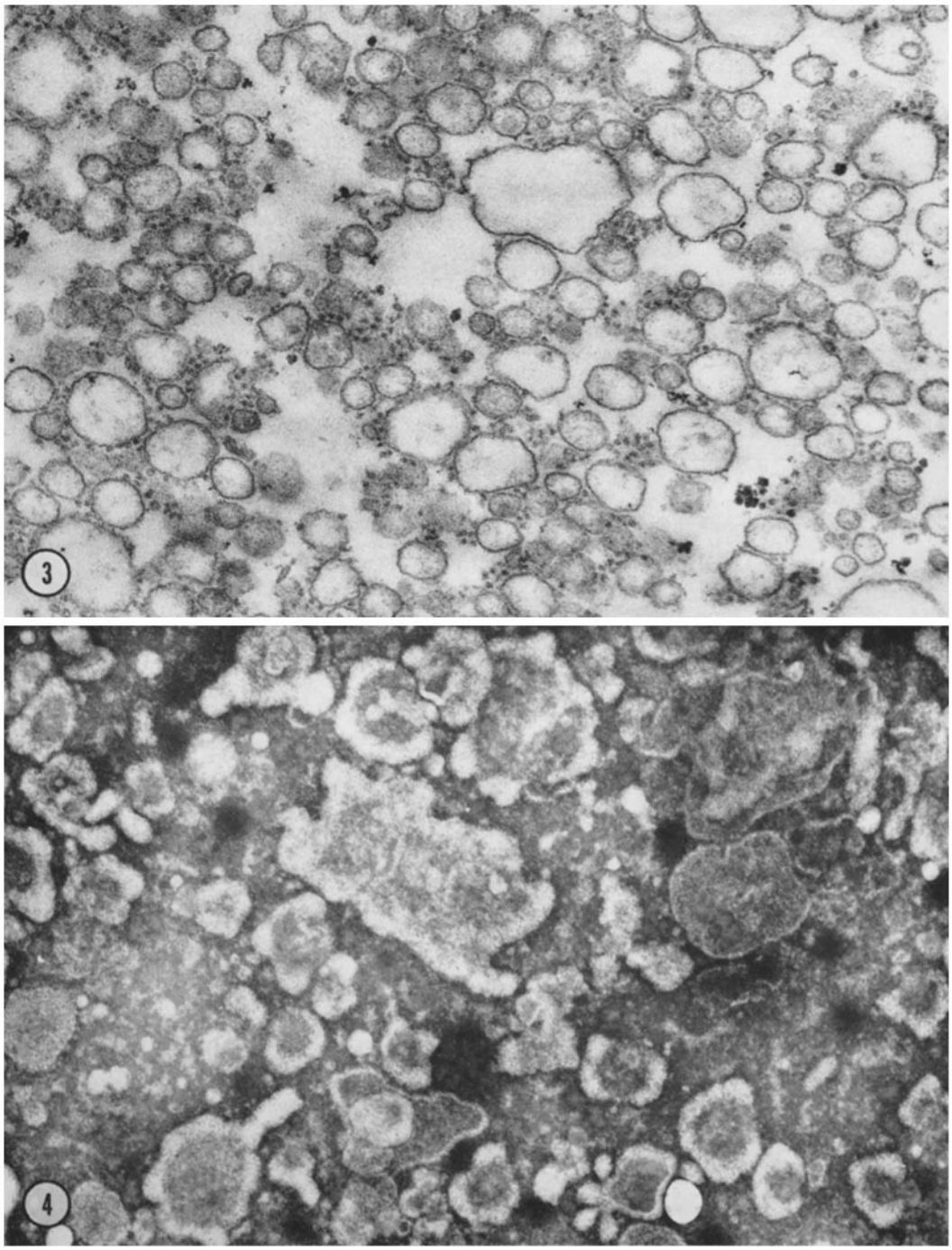
‡ Ratio of specific activity of endoplasmic reticulum–rich fraction to that of total homogenate ± average deviation.

vesicles of uniform size with attached ribosomes (Fig. 3). Preparations consistently had a fuzzy aspect both in thin section (Fig. 3) and in negative stain (Fig. 4). Much of the vesiculation occurred during the final resuspension in distilled water, and vesiculation was not observed to reduce the enzymatic activity of the preparations.

Golgi Apparatus–Rich Fraction

Golgi apparatus fractions described in the preceding report (17) were obtained from the sucrose gradients and analyzed directly. The results (Tables III, IV), which are derived from more than 100 routine preparations, each from a different animal, illustrate the range of variation encountered. The specific activity of 5′-nucleotidase of the Golgi apparatus–rich fraction was approximately 14% that of the plasma membrane fraction on a protein basis (Table IV). Glucose-6-phosphatase activity of the Golgi apparatus fraction ranged from 8–19% (average of about 10%) of that of purified endoplasmic reticulum fractions (Tables IV, V). Mitochondrial contamination of the Golgi apparatus fraction was low when measured by assays for either cytochrome oxidase or succinic-INT-reductase (1–3% that of washed mitochondria) (Tables IV, V). Uricase assays showed a maximum activity of 1% that of purified microbodies (Table IV). The relatively high specific activities of acid phosphatase reported for purified lysosomes (8) would suggest that the lysosomal contribution to total protein of the fraction would be in the same order as that for microbodies (Table III *a*). On this basis, no more than 25% of the protein of gradient-purified Golgi apparatus fractions appeared to be derived from the combined contribution of plasma membrane, endoplasmic reticulum, mitochondria, microbodies, and lysosomes (Table IV).

With further washing, these activities declined (Table V). With two additional resuspension-centrifugation cycles, 5′-nucleotidase activity declined by 50%, glucose-6-phosphatase activity by 80%, and succinate-INT-reductase activity by 25% relative to sucrose gradient purified fractions. With the use of the same basis of calculation as in Table IV, the maximum contribution of contaminating cell components to the total protein of



FIGURES 3 and 4 Endoplasmic reticulum–rich fraction from rat liver in thin section (Fig. 3, × 50,000) and negative contrast (Fig. 4, × 60,000).

TABLE III *a*

Specific and Relative Phosphorylitic Activity of the Golgi Apparatus-Rich Cell Fraction

Substrate	Specific activity*			Relative specific activity‡
	1	2	3	
	μmoles P_i/hr per mg protein			
Thiamine pyrophosphate	2.2	2.9	3.1	7.1 ± 0.4
β-Glycerol phosphate (pH 5.4)	0.7	0.6	0.6	3.4 ± 0.2
β-Glycerol phosphate (pH 9.0)	0.2	0.2	0.1	0.8 ± 0.1
Glucose-6-phosphate	1.4	1.2	0.8	0.7 ± 0.1
Adenosine-5′-monophosphate	5.0	6.1	6.5	3.9 ± 0.3
Cytidine-5′-monophosphate	3.2	1.7	4.8	2.2 ± 0.5
Inosine-5′-monophosphate	0.1	0.6	2.0	2.1 ± 0.3
Uridine-5′-monophosphate	3.1	3.5	3.4	2.5 ± 0.5
Guanosine-5′-monophosphate	1.7	1.7	3.2	2.2 ± 0.3
Thymidine-5′-monophosphate	1.5	2.4	1.7	1.7 ± 0.4
Adenosine-5′-diphosphate	1.4	2.2	1.6	1.9 ± 0.2
Cytidine-5′-diphosphate	2.0	2.7	0.4	2.6 ± 0.8
Inosine-5′-diphosphate	10.5	8.0	9.3	2.6 ± 0.3
Uridine-5′-diphosphate	0.2	1.1	4.8	2.5 ± 0.7
Guanosine-5′-diphosphate	8.9	5.7	5.6	2.4 ± 0.3
Thymidine-5′-diphosphate	0.8	0.8	1.4	1.6 ± 0.4
Adenosine-5′-triphosphate	1.6	3.4	2.6	2.9 ± 0.4
Cytidine-5′-triphosphate	0.4	0.6	2.0	3.4 ± 0.7
Inosine-5′-triphosphate	2.3	7.4	7.0	2.5 ± 0.3
Uridine-5′-triphosphate	1.7	1.3	3.5	2.3 ± 0.3
Guanosine-5′-triphosphate	6.1	11.1	4.0	3.0 ± 1.1
Thymidine-5′-triphosphate	1.6	1.4	3.4	2.2 ± 0.5

* Enzymatic activity was determined by the release of inorganic phosphate (P_i). Assay conditions were those of Emmelot et al. (10).

‡ Ratio of specific activity of the Golgi apparatus-rich fraction to that of total homogenate ± average deviation.

TABLE III *b*

Specific and Relative Oxido-Reductase Activities of the Golgi Apparatus-Rich Fraction

Enzyme	Specific activity*			Relative specific activity‡
	1	2	3	
Cytochrome oxidase	0.04	0.03	0.03	0.2 ± 0.0
Succinate-INT-reductase	0.07	0.20	0.22	0.1 ± 0.04
Uric acid oxidase	0.5	0.1	0.9	0.3 ± 0.1

* Units of specific activity are μmoles O_2/min per mg of protein for cytochrome oxidase; μmoles INT reduced/hr per mg of protein for succinate-INT-reductase; and μμmoles uric acid oxidized/min per mg of protein for uric acid oxidase.

‡ Ratio of specific activity of the Golgi apparatus-rich fraction to that of the total homogenate ± average deviation.

the Golgi apparatus fraction after two resuspension-centrifugation cycles would be 10% (Table V).

DISCUSSION

The methods of estimating contamination of the Golgi apparatus fraction (17) were similar to those employed previously for characterization of plasma membrane fractions (4, 6). The enzymes assayed are those generally accepted as biochemically typical of single cell components (4), and included 5′-nucleotidase for plasma membrane, succinic-INT-reductase, and cytochrome oxidase for mitochondria, glucose-6-phosphatase for endo-

TABLE IV

Relative Contribution of Cell Components to Golgi Apparatus–Rich Fraction

Enzyme	Fraction	Specific activity*	Ratio
5′-Nucleotidase (AMP)	Golgi apparatus	5.8	0.14
	Plasma membrane	41.9	
Glucose-6-phosphatase	Golgi apparatus	1.1	0.10
	Endoplasmic reticulum	11.4	
Cytochrome oxidase	Golgi apparatus	0.03	0.03
	Mitochondria	1.13	
Succinate-INT-reductase	Golgi apparatus	0.18	0.01
	Mitochondria	15.4	
Uric acid oxidase	Golgi apparatus	0.5	0.01
	Microbodies	50.0	

* Units as in Table III.

TABLE V

Effect of Further Purification on the Relative Contribution of Cell Components to Gradient-Purified Golgi Apparatus–Rich Fraction

	Relative specific activity			Relative contribution of contaminating cell component as % of total protein	
	No. of resuspension-centrifugation cycles			No. of resuspension-centrifugation cycles	
Enzyme	0	2	Cell component	0	2
5′-Nucleotidase (AMP)	4.5	2.3	Plasma membrane	11	6
Glucose-6-phosphatase	1.2	0.2	Endoplasmic reticulum	10	2
Succinate-INT-reductase	0.3	0.2	Mitochondria	2	1
			Total	23	9

plasmic reticulum, uricase for microbodies, and acid phosphatase for lysosomes. Localization of glucose-6-phosphatase with endoplasmic reticulum in our isolated preparations was verified at the electron microscope level by the method of Wachstein and Meisel (27) as modified by Schin and Clever (24). With all fractions, the degree of contamination by other cell components as estimated from enzymatic analyses was verified or confirmed as an upper limit by examination of the preparations with the electron microscope (Figs. 1–4, reference 17).

Estimates of the degree of purity, assessed on a specific activity basis, are dependent on the purity and the relative protein contents of the fractions compared. Our Golgi apparatus and plasma membrane fractions contain about 60% of the dry weight as protein, whereas our endoplasmic reticulum and mitochondrial fractions contain approximately 70% of the dry weight as protein. We have also assumed completely specific localization of the marker enzymes in a particular cell component. All three considerations, lack of homogenous reference fractions, small differences in protein content, and lack of specificity, however, lead to an underestimation rather than an overestimation of fraction purity. Thus, our estimates of the contribution of other cell components to the Golgi apparatus fraction represent an upper limit, and the purity of the fractions may be somewhat higher than the calculations indicate.

Other considerations affecting the interpreta-

tion of the results include differences in preparative procedures which contribute to leakage of enzymes, nonspecific binding, denaturation, activation, and other phenomena that selectively alter the enzymatic activity of one preparation relative to another. For partially circumventing these difficulties, Golgi apparatus, endoplasmic reticulum, and mitochondrial fractions were isolated by means of the same homogenization procedure. Variations due to individual animals were normalized by determining specific activities relative to the total homogenate. This latter method was also employed with plasma membrane analyses where a different homogenization technique was used. Since preparation of endoplasmic reticulum required a lengthy centrifugation step, we analyzed all material derived from the original homogenate for glucose-6-phosphatase to show quantitative recovery on a specific activity basis.

In evaluating the results of these enzymatic analyses, the heterogeneous nature of the Golgi apparatus–rich fraction must be considered. Different membrane types and structures are present and include secretory vesicles, tubules and cisternae (17). Some of the enzymatic activity ascribed to contaminating cell components might be associated with these functionally specialized portions of the Golgi apparatus, i.e. glucose-6-phosphatase with peripheral tubules adjacent to rough endoplasmic reticulum or 5′-nucleotidase with secretory vesicle membranes (17). That the Golgi apparatus fraction contained acid phosphatase (Table III) is not unexpected since lysosome-like vesicles are commonly observed in the Golgi apparatus preparations (17), and lysosomes are known to originate as secretory vesicles derived from the Golgi apparatus (11). It is doubtful that the mitochondrial electron-transport enzymes are a part of the Golgi apparatus although nonspecific binding of these enzymes (or of other enzymes) is not ruled out.

Of the enzyme activities reported here, none can be considered unique to the Golgi apparatus fraction (Table III). Thiamine pyrophosphatase (TPPase) has been localized within the Golgi apparatus for a number of cell types including liver by ultrastructural cytochemistry (9, 19, 20). At a near optimum pH of 8.0 (1), the specific activity of the Golgi apparatus fraction was about seven times that of the total homogenate, but the catalysis of TPP hydrolysis by other cell fraction from rat liver was sufficient to complicate use of this enzyme as a specific in vitro marker for Golgi apparatus with our preparations.

Nucleoside di- and triphosphates were all cleaved by the Golgi apparatus fractions at pH 7.4 (Table III). Without exceptions, the activity of the Golgi apparatus fraction was intermediate between that of the endoplasmic reticulum- and plasma membrane–rich fractions (Cheetham, unpublished). However, since assay conditions were not varied, this is probably an oversimplification of a complicated pattern of enzyme distribution. For example, the extensive cytochemical studies of Novikoff and coworkers (13, 19, 20) show certain nucleoside diphosphatase activities to be specifically localized in Golgi apparatus membranes. In addition to the activities reported in Table III, the Golgi apparatus fraction contains choline kinase activity and CDP-choline-cytidyl transferase activities (18), although these enzymes are not specifically localized there.

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