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SEQUENTIAL PREPARATION OF RAT LIVER MICROSOMAL AND GOLGI MEMBRANES

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Summary

A new procedure for the preparation of microsomes, microsomal subfractions and Golgi membranes from the same piece of rat liver has been developed. The smallest amount of liver with which the preparation can be performed is about 1 g (wet weight).

35% of the total activity of marker enzymes for the endoplasmic reticulum was recovered in the microsomal fraction. This recovery is approximately the same as that obtained in our laboratories using other procedures. Golgi membranes, mitochondria, lysosomes and plasma membranes represent less than 13% of the microsomal protein as calculated on the basis of marker enzymes.

Golgi membranes must be prepared in two steps to achieve a reasonable recovery and thus a representative sample containing both very low density lipoprotein-rich Golgi vesicles and the heavier cisternal elements. The recovery of UDP-galactosyltransferase activity in the Golgi fraction from the livers of alcohol-treated animals is around 30% of the total activity in the total particulate fraction.

Introduction

In the field of experimental biology there is a need for techniques for preparing pure and representative samples of subcellular organelles from small pieces of tissue. This is especially important when studying experimental and clinical pathologies, for example, in studies of chemical carcinogenesis, where each animal develops an unique population of tumor cells. Furthermore, for complicated research procedures such as use of tissue perfusion to investigate biogenetic pathways by following the incorporation of radioactively-labeled compounds into different organelles, and in examination of human tissues

where often only restricted biopsy material is accessible, suitable fractionation procedures have not been available. In this study we have developed a new technique for preparation of microsomes and submicrosomal fractions as well as Golgi vesicles with good recovery and high purity. The method is simple and can be completed within one working day. All procedures are performed using commercially available centrifuges and rotors, which make the method convenient for general use.

Materials and Methods

Male adult Sprague-Dawley rats weighing 180—200 g were fasted for 20 h before decapitation. 90 min before decapitation the rats were given 50% ethanol (1.2 ml/100 g body weight) through a stomach tube, according to Ehrenreich et al. [1].

After decapitation the livers were removed and minced carefully in 0.25 M sucrose using a pair of scissors. All preparative work was performed at 4°C. The liver pieces were rinsed in cold sucrose 5 times in order to remove blood and connective tissue debris. The small pieces of liver were then transferred to a Potter-Elvehjem glass Teflon homogenizer and homogenized with 3 strokes at a pestle speed of 885 rev./min. The concentration of the homogenate was adjusted to 1 g liver/5 ml 0.25 M sucrose. The total particulate fraction was prepared by centrifuging the tissue homogenate at 105 000 × *g* for 90 min and the pellet was resuspended in 0.25 M sucrose to a concentration corresponding to 1 g liver/20 ml and then sonicated in an MSE sonifier at 1.5 A for 5 min in intervals of 30 s while maintaining the temperature between 2 and 8°C.

In order to obtain the postmitochondrial supernatant the homogenate was centrifuged at 10 000 × *g* for 20 min in an 8 × 50 angle rotor (MSE High speed). Microsomes were prepared from this supernatant by spinning at 105 000 × *g* for 90 min, after which the pellet was resuspended in 0.25 M sucrose to a final concentration equivalent to 1 g liver/ml.

For the fractionation of rough, smooth and Golgi I membranes discontinuous sucrose gradients were formed as shown in Fig. 1. 4 ml of the 10 000 × *g* supernatant, which corresponds to 1 g liver, was layered onto a discontinuous sucrose gradient composed of 0.5 ml of 0.6 M sucrose and 2 ml of 1.3 M sucrose, both containing 15 mM CsCl. After centrifugation at 105 000 × *g* for 90 min in a 40.2 rotor (LKB Beckman) the smooth plus Golgi I fraction was

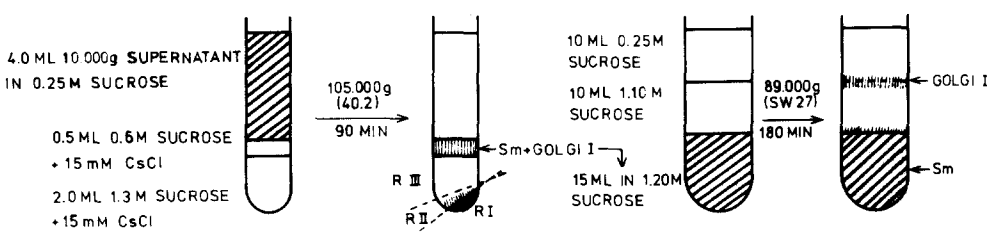

Fig. 1. Schematic representation of the procedure for preparation of microsomal subfractions and Golgi membranes. R I, R II, R III, rough I, II and III; Sm, smooth.

collected using a 5 ml syringe attached to a bent needle. The tight pellet containing ribosome-rich rough microsomes was resuspended in 0.25 M sucrose and called rough I [2]. The ribosome-poor fractions R II and R III were sucked off and discarded and were not further studied in this paper but can be easily collected using this method of preparation.

2.1 M sucrose was added to the smooth plus Golgi I fraction in sufficient amount to give a final sucrose concentration of 1.20 M. The sucrose concentration was checked using a Zeiss refractometer. Usually the preparation was performed using 12 g liver tissue, wet weight. In these situations the SW 27 rotor was used and the gradients were built up as follows. 15 ml of the suspension above was pipetted into an SW 27 tube and 10 ml 1.10 M sucrose was subsequently layered on top. Finally, 10 ml 0.25 M sucrose was carefully layered to fill the tube. The gradient was centrifuged at 89 000 × *g* for 180 min in an SW 27 rotor (LKB Beckman). Starting with the minimal amount of tissue, 1 g liver, i.e., material from one 40.2 tube, the SW 40 rotor was used for the floatation procedure. To the smooth plus Golgi I fraction was added 2.1 M sucrose to yield the final sucrose concentration of 1.20 M. The obtained suspension, around 2 ml, was pipetted into a SW 40 tube and 5 ml of 1.10 M sucrose was layered above. The tube was filled by layering 0.25 M sucrose on top. The gradient was thereafter centrifuged at 90 000 × *g* for 180 min in an SW 40 rotor. The Golgi I fraction was collected from the 1.10 M/0.25 M sucrose interface while the smooth fraction remained in the 1.20 M sucrose. The smooth and Golgi fractions were pelleted at 105 000 × *g* for 90 min and subsequently suspended in 0.25 M sucrose.

For preparation of Golgi II membranes the 10 000 × *g* pellet was gently resuspended by hand in 0.25 M sucrose using a glass-Teflon homogenizer of the Potter-Elvehjem type [3]. The suspension was centrifuged at 10 000 × *g* for 10 min, and the membranes in the supernate were pelleted, resuspended in sucrose, adjusted to a sucrose concentration of 1.20 M, and used as starting material for the floatation procedure described above. In this case the 1.10 M sucrose was replaced by 1.15 M and the Golgi II fraction was collected at the 1.15 M/0.25 M sucrose interface.

All centrifugation procedures were performed with a Beckman L5-65 ultracentrifuge. The Golgi I and II pellets were resuspended in 0.25 M sucrose to a concentration corresponding to 5 g liver/ml and the smooth fraction was resuspended to 1 g liver/ml.

Plasma membranes were prepared according to Coleman et al. [4], lysosomes according to Leighton et al. [5], and mitochondria according to Sottocasa et al. [6].

Where indicated the fractions were suspended in 0.15 M Tris-HCl, pH 8.0, and subsequently centrifuged at 105 000 × *g* for 90 min. These pellets were suspended in sucrose and are referred to as washed membranes.

Chemical analysis. Protein was measured according to the method of Lowry et al. [7] with bovine serum albumin as a standard. RNA was measured using the orcinol method [8] with ribose as a standard. Phospholipids were determined by the method of Marinetti [9].

Enzymic assays. The cytochrome *c* reductases and the phosphatases were assayed immediately after preparation of the fraction to obtain maximal activ-

ity. The activities of NADPH- and NADH-cytochrome *c* reductase and glucose-6-phosphatase were measured as described previously [2], while the activities of adenosine 5'-monophosphatase, succinate-cytochrome *c* reductase and β-glycerophosphatase were measured according to Beaufay et al. [10]. The activity of UDPgalactosyltransferase was measured using desialidated mucin as an exogenous acceptor [10,11]. Before analyzing UDPgalactosyltransferase the membranes were washed in 0.15 M Tris-HCl buffer, pH 8.0, as described above, in order to remove adsorbed cytosolic and serum hydrolytic enzymes such as galactosidases and pyrophosphatases.

Results

The chemical compositions of microsomal and Golgi membranes prepared by a two-step centrifugation procedure (including a discontinuous cation-containing sucrose gradient for the preparation of rough and smooth membranes and a floatation procedure for the preparation of Golgi membranes I and II) are shown in Table I. After homogenization with 3 strokes at 885 rev./min in a glass-Teflon homogenizer, the recovery of microsomal protein is about 23 mg/g liver, wet weight. The two main subfractions of microsomes which were prepared in this study are the rough I and smooth microsomes, containing 10.1 and 5.0 mg protein/g liver, respectively. The phospholipid per protein ratio is almost the same in the microsomes and the microsomal subfractions. 70% of the RNA in the microsomes is recovered in the rough I microsomal fraction, while 13% is found in the smooth fraction. However, 68% of the RNA in the smooth fraction is located in free ribosomes not associated with the membrane. This was shown by rate differential centrifugation as described by Dallner and Nilsson [12], where the larger membrane vesicles form a pellet and the smaller ribosomes remain in the supernate. Further information concerning the distribution of free ribosomes in this type of gradient is found in ref. 2.

The Golgi I fraction prepared by floatation of the first postmitochondrial supernate contains vesicles with a density of 1.146 or lower. The recovery of protein in this fraction amounts to 0.51 mg/g liver. The Golgi II fraction, pre-

TABLE I

CHEMICAL COMPOSITION OF MICROSOMAL AND GOLGI MEMBRANES

The fractions were prepared as described in Materials and Methods.

	Protein (mg/g liver)	Protein washed * (mg/g liver)	Phospho-lipids (mg/g liver)	RNA (mg/g liver)	Phospholipid / protein	RNA / protein
Total particulate fraction	158.6	85.0	34.74	8.79	0.22	0.055
Microsomes	23.2	13.65	8.0	2.97	0.34	0.13
Rough I microsomes	10.1	5.31	3.06	2.06	0.30	0.20
Smooth microsomes	5.0	2.68	1.94	0.38	0.39	0.076
Golgi I	0.51	0.31	0.28	0.006	0.55	0.012
Golgi II	0.58	0.27	0.26	0.009	0.45	0.016

* The membranes were suspended once in 0.15 M Tris-HCl, pH 8.0, and centrifuged at 105 000 × *g* for 90 min.

pared from the second postmitochondrial supernatant, contains 0.58 mg protein/g liver. The phospholipid contents of these vesicles are high, but most of the lipids are localized in luminal very low density lipoproteins [1,13].

The distribution of microsomal and Golgi marker enzymes is illustrated in Table II. Based on the glucose-6-phosphatase and NADPH-cytochrome *c* reductase activities about 35% of the endoplasmic reticulum is recovered in the microsomes. This figure is in agreement with those earlier published [3,14—16].

All the microsomal marker enzymes in Table II show the same distribution pattern in the microsomal fraction and microsomal subfractions, which is strong evidence that their recovery is a good indication of the recovery of endoplasmic reticulum membranes. The activities of glucose-6-phosphatase and NADPH-cytochrome *c* reductase in the Golgi I and II fractions correspond to a calculated microsomal 'contamination' of 12—15% of the protein in the Golgi fractions. The specific activities of microsomal marker enzymes in Golgi fractions with a density of 1.085 or lower indicate substantially lower contamination, i.e. 5—7% (not shown in table). The degree of contamination has been calculated by dividing the specific activities of microsomal marker enzymes in the Golgi fractions by the specific activities of these enzymes in the microsomal fraction and is expressed as the percent of the protein in the Golgi fraction originating from the endoplasmic reticulum.

The activity of UDPgalactosyltransferase measured using desialidated mucin as an exogenous acceptor in Golgi I is 53 pmol galactose transferred/min per g liver, i.e., 15% of the activity in the total particulate fraction. In Golgi II the corresponding figures are 46 pmol galactose transferred/min per g liver and 13% of the total. Taken together, these two Golgi fractions correspond to a recovery of about 30%. This relatively low recovery is the price which must be paid for obtaining Golgi and microsomal membranes of high purity, as will be discussed later. The activity of UDPgalactosyltransferase in rough I and smooth microsomes is low. If this activity is due only to Golgi membrane contamination, it would represent 0.6% and 3.5% of the protein in the rough I and smooth fractions, respectively.

To estimate the degree of contaminating membranes in the different subfractions we have measured a number of 'marker enzymes' for mitochondria (monoaminoxidase, succinate-cytochrome *c* reductase and cytochrome *c* oxidase), lysosomes (β-glycerophosphatase, β-galactosidase and cathepsin D) and plasma membranes (AMPase). For each organelle the activities of the different enzymes showed parallell distribution patterns among the subfractions. We have, in Table III, chosen to present the results obtained for succinate-cytochrome *c* reductase, β-glycerophosphatase and AMPase, which are all well known and generally accepted markers for mitochondrial outer membrane [6,17], lysosomes [5] and plasma membranes [10], respectively. The activities of these enzymes in the different subfractions have been compared with the specific activities of the enzymes in purified preparations of mitochondria, lysosomes and plasma membranes. These reference preparations are not perfectly pure and concerning lysosomes and plasma membranes the recovery is low, facts which allow one to assume that the specific activities of the relevant markers are relatively low in these fractions. The amount of contaminating pro-

TABLE II

THE ACTIVITIES OF MICROSOMAL AND GOLGI MEMBRANE MARKER ENZYMES

Fractions prepared as described in Materials and Methods. The values are the mean of 6 experiments.

	NADPH-cytochrome *c* reductase		NADH-cytochrome *c* reductase		Glucose-6-phosphatase		UDPgalactosyltransferase	
	abs. act. [a]	spec. act. [b]	abs. act. [a]	spec. act. [b]	abs. act. [c]	spec. act. [d]	abs. act. [e]	spec. act. [f]
Total particulate fraction	7.65	0.048	49.00	0.31	20.67	0.13	351	4.14
Microsomes	2.85	0.12	16.14	0.69	7.76	0.33	76	5.57
Rough I microsomes	1.05	0.10	4.21	0.42	3.53	0.35	6	1.13
Smooth microsomes	0.86	0.17	4.17	0.79	1.4	0.28	18	6.72
Golgi I	0.008	0.016	0.041	0.08	0.020	0.04	53	171.0
Golgi II	0.010	0.018	0.062	0.11	0.028	0.05	46	170.0

[a] μmol cytochrome *c* reduced/min per g liver.
[b] μmol cytochrome *c* reduced/min per mg protein.
[c] μmol inorganic phosphorus released/min per g liver.
[d] μmol inorganic phosphorus released/min per mg protein.
[e] pmol galactose transferred/min per g liver.
[f] pmol galactose transferred/min per mg protein (washed).

TABLE III

DISTRIBUTION OF SOME ENZYMES IN MICROSOMAL AND GOLGI MEMBRANES

Fractions prepared as described in Materials and Methods. The values are the mean of 5 experiments.

	Succinate-cytochrome *c* reductase		β-Glycerophosphatase		AMPase	
	abs. act. [a]	spec. act. [b]	abs. act. [c]	spec. act. [d]	abs. act. [c]	spec. act. [d]
Total particulate fraction	3.93	0.025	9.56	0.060	8.23	0.052
Microsomes	0.16	0.007	0.84	0.036	0.74	0.032
Rough I microsomes	0.055	0.005	0.33	0.033	0.24	0.024
Smooth microsomes	0.003	0.0006	0.17	0.034	0.24	0.048
Golgi I	0	0	0.077	0.15	0.092	0.18
Golgi II	0	0	0.018	0.03	0.30	0.51
Mitochondria	3.53	0.15				
Lysosomes			2.7	2.55		
Plasma membranes					0.50	0.89

[a] μmol cytochrome *c* reduced/min per g liver.
[b] μmol cytochrome *c* reduced/min per mg protein.
[c] μmol inorganic phosphorus released/min per g liver.
[d] μmol inorganic phosphorus released/min per mg protein.

tein in microsomal and Golgi fractions shown in Table III is calculated on these data and must therefore be regarded as substantially higher than the actual one.

Table III shows the distribution of succinate-cytochrome *c* reductase, β-glycerophosphatase and AMPase. By comparing the specific activity of succinate-cytochrome *c* reductase in the purified mitochondrial preparation with the activities in the microsomal and Golgi fractions, the contamination of mitochondrial protein in rough I and smooth microsomes can be calculated to be 3.3 and 0.4%, respectively. The amount of mitochondrial protein in the Golgi fraction is negligible.

The absolute activity of β-glycerophosphatase in rough I and smooth microsomes amounts to 3.5 and 1.8% of that in the total particulate fraction. If the presence of this enzyme in the microsomal fractions is to be regarded as due solely to contamination, lysosomes should make up 1.2% of the protein in both fractions. In the Golgi I fraction the specific activity of β-glycerophosphatase is 5 times that in microsomes, while in the Golgi II fraction it is the same as the activity in microsomes. Farquhar et al. [18], as well as Widnell [19] have postulated that acid phosphatases occur endogeneously in some of the Golgi vesicles, preferentially the light, lipid-rich GF_1 and GF_2 fractions. Novikoff et al. [20] have also demonstrated acid phosphatases in Golgi vesicles from exocrine pancreas cells. In our opinion it is therefore not reasonable to calculate the lysosomal contamination in the Golgi fractions using acid phosphatase as marker enzyme. Anyhow, using this enzyme as a lysosome marker the contamination should be as low as 1—2% of the protein. (For further comments, see Discussion).

AMPase activity is widely distributed among cellular organelles, including the endoplasmic reticulum and Golgi membranes [18,19], but is present at highest specific activity in the plasma membrane. The absolute activity of AMPase in microsomes amounts to 9% of that in total particulate fraction, corresponding to a 3.6% contamination of plasma membrane protein. Taking into account the possibility that AMPase is located in other membranes than the plasma membrane and using the AMPase activity of pure rough microsomal membranes as a measure of microsomal endogeneous AMPase, the degree of microsomal contamination by plasma membrane amounts to 1% of the protein. In Golgi I membranes the AMPase activity is 0.12 μmol P_i released/min per mg protein, while in Golgi II it is 0.51 μmol. There is much evidence in the literature that AMPase is heterogeneously distributed between the different Golgi vesicles with the highest activities in the heavy Golgi fraction [13,18]. The activity of this enzyme should therefore not be used to determine plasma membrane contamination.

As an indicator of the possible effect of ethanol treatment on the physicochemical properties of microsomal membranes the effect of sodium deoxycholate on the activity of glucose-6-phosphatase was investigated. It has been reported that deoxycholate enhances the permeability of the membrane and thereby increases the activity of the enzyme [21—24]. Thus, if maximal glucose-6-phosphatase activity is obtained even without detergent, this would indicate that the membranes are freely permeable for the substrate and should thereby be regarded as more or less disintegrated. In Table IV the effects of deoxycholate on the activity of glucose-6-phosphatase in microsomal subfrac-

TABLE IV

THE EFFECT OF DEOXYCHOLATE ON THE ACTIVITY OF GLUCOSE-6-PHOSPHATASE IN ROUGH AND SMOOTH MICROSOMES FROM CONTROL AND ALCOHOL-TREATED RATS

Preparations were performed as described in Materials and Methods. The values are the mean of 4 experiments.

	Glucose-6-phosphatase			
	Detergent [c]		No detergent	
	abs. act. [a]	spec. act. [b]	abs. act. [a]	spec. act. [b]
Control				
Rough microsomes	3.33	0.34	1.86	0.19
Smooth microsomes	1.43	0.28	0.77	0.15
Alcohol treatment [d]				
Rough microsomes	3.53	0.35	2.07	0.20
Smooth microsomes	1.40	0.28	0.87	0.17

[a] μmol P_i released/min per g liver.
[b] μmol P_i released/min per mg protein.
[c] Sodium deoxycholate at a final concentration of 0.03%.
[d] 2 ml 50% ethanol was instilled in the ventricle 90 min before decapitation.

tions from alcohol-treated and control rats are presented. In both fractions the enzyme activity is submaximal without detergent and is increased by around 40% when deoxycholate is added to the incubation mixture. The results are identical whether alcohol was used or not, supporting the idea that alcohol does not damage microsomal membranes in this respect.

Table V shows the recovery of microsomes and Golgi membranes when preparations were performed without alcohol pretreatment. In these experiments the recovery of Golgi membranes is substantially lower than in the case of alcohol treatment, but the specific activity of UDPgalactosyltransferase is as high as in alcohol-treated rats. Furthermore the activity in smooth microsomes of UDPgalactosyltransferase is higher in the control rats, which is taken as an

TABLE V

THE RECOVERY OF PROTEIN AND THE DISTRIBUTION OF GALACTOSYLTRANSFERASE IN MICROSOMAL AND GOLGI MEMBRANES PREPARED FROM CONTROL AND ALCOHOL-TREATED RATS

The fractions were prepared as described in Materials and Methods with the exception of the control rats where no alcohol was instilled in the ventricle. The values are the mean of 3 experiments.

	Control		Alcohol treated	
	Protein * (mg/g liver)	UDPgalactosyltransferase (pmol galactose transferred/ min per mg protein)	Protein * (mg/g liver)	UDPgalactosyltransferase (pmol galactose transferred/ min per mg protein)
Rough I	5.18	1.09	5.31	1.13
Smooth	2.59	8.27	2.68	6.72
Golgi I	0.19	176.5	0.31	171.0
Golgi II	0.21	204.7	0.27	170.0

* The membranes were washed once in 0.15 M Tris-HCl, pH 8.0, as described previously.

indication that a considerable amount of Golgi vesicles remain in the smooth microsomal fraction.

Discussion

Isolation of representative parts of the endoplasmic reticulum and the Golgi membranes from the same piece of liver is very important in experimental as well as clinical pathology. In such studies the availability of material is often very restricted and there is a necessity of getting as much information as possible from the sample. The investigations presented in this paper show that it is possible to fractionate microsomes and microsomal subfractions as well as Golgi membranes and subfractions in a sequential procedure from a single piece of liver tissue. The recovery and purity of rough I and smooth microsomes are identical with those obtained when the tissue is used for preparation of these membrane fractions exclusively [3,14—16] and it has been shown that the microsomal and submicrosomal vesicles prepared with this method are chemically and enzymatically representative of the total endoplasmic reticulum [3]. Furthermore, by a simple resuspension of the 10 000 × *g* pellet followed by differential centrifugation the recovery of endoplasmic reticulum membranes can be nearly double (see ref. 3).

The preparation of Golgi membranes must be performed in two steps to achieve acceptable recovery and a reasonably representative sample. The Golgi I fraction is prepared from the material in the supernatant obtained after centrifugation at 10 000 × *g* for 20 min. This Golgi fraction corresponds mainly to the GF_1 and GF_2 fractions obtained by the procedure described by Ehrenreich et al. [1]. To obtain the heavier Golgi vesicles (Golgi II), the 10 000 × *g* pellet was gently resuspended and recentrifuged at 10 000 × *g* for 10 min. The particulate material in this supernate with a density of 1.146 or lower was collected. This material exhibited the same high specific activity of UDPgalactosyltransferase as shown by Bergeron et al. [13] in the GF_3 fraction.

The activity of β-glycerophosphatase and AMPase in the Golgi I and Golgi II fractions shows a characteristic distribution in agreement with data found by Bergeron et al. [13] and Farquhar et al. [18] using both biochemical and histochemical techniques. Considerable amounts of acid phosphatases (which is not to be regarded as solely lysosomal) can be demonstrated within the light vesicles with a density of 1.116 or lower. AMPase activity has been demonstrated in vesicles corresponding to the GF_3 Golgi fraction. Thus, the high activity of AMPase in our Golgi II fraction probably reflects the activity in the Golgi membranes themselves. The high specific activity of UDPgalactosyltransferase makes plasma membrane contamination highly improbable.

Alcohol treatment induces a number of well known alterations in liver lipid, protein and carbohydrate metabolism, some of which are associated with the endoplasmic reticulum and Golgi membranes [25]. To determine whether treatment had any effect on the permeability properties of microsomal vesicles, we used the system first employed by de Duve et al. [21] and later described by Arion et al. [22,23] and Nilsson et al. [24], where the activity of glucose-6-phosphatase was measured in the absence and presence of detergent. We found the same glucose-6-phosphatase activity and latency in microsomes from con-

trol and alcohol-treated animals. The results are in agreement with earlier studies.

Preparations were also performed without alcohol pretreatment. These studies resulted in a 35% less recovery of Golgi membranes and a considerably higher contamination of the smooth fraction by Golgi marker enzymes. Anyhow, the fractions are in all respects acceptable for further studies, bearing in mind the low recovery of Golgi membranes and the limitations of the smooth fraction concerning Golgi contamination.

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