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# PREPARATION OF LIVER MICROSOMES WITH HIGH RECOVERY OF ENDOPLASMIC RETICULUM AND A LOW GRADE OF CONTAMINATION

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## Summary

A modified procedure for preparing the microsomal fraction from rat liver was developed with the aim of increasing the recovery without increasing the degree of contamination.

87% of the membranes of the microsomal fraction isolated from the first mitochondrial (10000 $\times g$) supernatant originates from the endoplasmic reticulum, representing a 35% yield. By gentle resuspension of the 10000 $\times g$ pellet followed by differential centrifugation a second crop of microsomes can be prepared which, together with the first crop, gives a 55% total recovery of microsomal markers. 87% of the protein in this second crop also originates from the endoplasmic reticulum and this fraction has properties similar to those of the first crop. Contaminating membranes include Golgi membranes (0.6% of the total protein), mitochondria (2.5%), lysosomes (5%) and plasma membranes (5%). Collecting further crops increases the contamination.

Subfractionation studies revealed almost identical distributions of ribosome-rich, ribosome-poor and smooth membranes in the two crops of microsomal fractions.

The results obtained after treatment of the animals with phenobarbital or methylcholantrene were similar to those obtained with control animals; but in the case of methylcholantrene treatment the second crop represents a larger portion of the total membranes of the endoplasmic reticulum.

## Introduction

Investigations on the structure and function of the endoplasmic reticulum requires the isolation of this organelle with as high a recovery and as high purity as possible. Analyses of the microsomal fraction prepared in different laboratories have conclusively demonstrated that it is very difficult to achieve

both of these aims [1—4]. Disruption of all hepatocytes requires vigorous and repeated mechanical treatment, since this is the only way to liberate all the intracellular organelles. On the other hand, such intensive homogenization results in disruption of various cellular membranes such as plasma membranes, Golgi vesicles, lysosomes and outer mitochondrial membranes, as well as in disruption of non-parenchymal cells. Consequently, effective homogenization always results in isolation of highly contaminated microsomal fractions.

Relatively mild homogenization appears to disrupt a large portion of the hepatocytes and this breakage appears to take place in a random manner. The main problem arises during removal of large particles and intact cells by low speed centrifugation. During this centrifugation a sizeable portion of the microsomal vesicles are found in the pellet because of entrapment and because of the nature of differential centrifugation [5,6]. It is obvious that a high recovery can be attained by isolation not only of the microsomal vesicles remaining in the low-speed supernatant, but by the recovery of those vesicles which are usually lost in the first low speed centrifugation as well. However, a basic requirement for such a procedure is that contamination from membranes other than those of the endoplasmic reticulum not be increased. Thus, a relatively high recovery should be attained while maintaining high purity.

In this study we have worked out a procedure for collecting a substantial portion of the microsomal vesicles lost during the first low speed centrifugation without breaking up other organelles, which would result in increased contamination.

## Materials and Methods

Adult male albino rats weighing 180—200 g were fasted for 20 h before killing. Where indicated, phenobarbital (8 mg/100 g body weight, dissolved in sterile Ringer's solution) was administered intraperitoneally once daily for 4 days or 3-methylcholanthrene (2 mg/100 mg body weight, dissolved in corn oil) 5 and 3 days before decapitation.

For preparation of liver homogenate rats were sacrificed by decapitation and the livers removed and minced carefully in 0.25 M sucrose at 0°C using a pair of scissors. The small pieces of liver were rinsed four times in cold sucrose and were thereafter transferred to a Potter-Elvehjem glass-teflon homogenizer and homogenized, in 5 ml 0.25 M sucrose/g liver wet weight, using four strokes at a pestle speed of 400 rev./min. To avoid heat denaturation of cellular enzymes, as well as disruption of membranes, all preparative procedures were performed at 4°C. The resulting homogenate contains intact cells, large particles and connective tissue, and therefore, for estimation of the total activity of marker enzyme the homogenate was diluted to contain material from 1 g of liver per 20 ml 0.25 M sucrose and sonicated in a Branson sonifier at 1.5 A for 2 min. This procedure results in a homogeneous and not too viscous preparation, here called total homogenate. The total particulate fraction was prepared by spinning the total tissue homogenate at 105000 $\times g$ for 90 min and thereafter resuspended in 0.25 M sucrose (20 ml/g liver wet weight) and sonicated as above.

The 600 $\times g$ supernatant was the supernate obtained after centrifugation of the homogenate at 600 $\times g$ for 10 min. This supernatant was sonicated as described above.

For preparation of the various microsomal fractions, livers were minced and homogenized as described above; the homogenate was centrifuged in a JA-20 rotor (JA-21 centrifuge, LKB-Beckman) at 10000 $\times g$ for 20 min and the supernatant was subsequently centrifuged at 105000 $\times g$ for 90 min in an L2-65 B centrifuge (LKB-Beckman). The pellet containing microsomes was resuspended in 0.25 M sucrose and called microsomes I. The 10000 $\times g$ pellet was gently resuspended in 0.25 M sucrose (5 ml/g of liver wet weight) using a glass-teflon homogenizer by moving the pestle 4 times up and down by hand. This suspension was centrifuged as above at 10000 $\times g$ for 20 min, and the supernatant used for preparation of microsomes II by centrifugation at 105000 $\times g$ for 90 min. Microsomes III were prepared in a manner similar to microsomes II, using the 10000 $\times g$ pellet obtained during preparation of microsomes II. Microsomes IV were prepared in an analogous manner.

Preparation of microsomal subfractions on cation-containing discontinuous sucrose gradient was performed as described previously [7,8], starting with the 10000 $\times g$ supernatant from the original differential centrifugation containing microsomes I and from the 10000 $\times g$ supernatant containing microsomes II. Proteins, phospholipids and RNA were measured as described earlier [7,8]. The activities of NADPH-cytochrome *c* reductase, and glucose-6-phosphatase were measured as described previously [7] while the activities of adenosine 5'-monophosphatase, succinate-cytochrome *c* reductase and $\beta$-glycerophosphatase were measured according to Beaufay et al. [9]. The CMP-sialyltransferase was measured using desialidated fetuin as exogeneous acceptor [9,10].

## Results

As a result of differential centrifugation, small particles, which at the start of the centrifugation are localized near or even in the volume later to be occupied by the pellet, will be entrapped in the pellet after centrifugation. Using repeated resuspensions followed by differential centrifugations at 10000 $\times g$ for 20 min it is possible to recover more and more of the entrapped smaller particles (in this case the microsomes) in the supernatant. Table I summarizes the recovery values for microsomes I, II, III and IV. As can be seen from the table, microsomes II contain 70—75% as much protein, phospholipid, and RNA

TABLE I

CHEMICAL COMPOSITION OF MICROSOMAL FRACTIONS OBTAINED BY RESUSPENSION OF THE 10 000 $\times g$ PELLET

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

| | Protein | Phospholipids (mg/g liver) | RNA | RNA / Protein |
|---|---|---|---|---|
| Total particulate fraction | 154.2 | 30.3 | 8.7 | 0.06 |
| Microsomes I | 19.8 | 6.2 | 2.3 | 0.12 |
| Microsomes II | 14.1 | 4.6 | 1.6 | 0.11 |
| Microsomes III | 9.6 | 3.1 | 0.9 | 0.09 |
| Microsomes IV | 6.1 | 1.9 | 0.6 | 0.10 |

TABLE II

DISTRIBUTION OF NADPH-CYTOCHROME *c* REDUCTASE AND GLUCOSE-6-PHOSPHATASE ACTIVITIES IN MICROSOMAL FRACTIONS

The total homogenate was sonicated for 2 min before enzyme analyses. The 600 × *g* supernatant was prepared by centrifuging the total homogenate at 600 × *g* for 10 min, followed by sonication of the resulting supernatant for 2 min. Otherwise as in Table I.

| | NADPH-cytochrome *c* reductase | | Glucose-6-phosphatase | |
|---|---|---|---|---|
| | Absolute activity (μmol cytochrome *c* reduced/min per g liver) | Specific activity (μmol cytochrome *c* reduced/min per mg protein) | Absolute activity (μmol $P_i$/min per g liver) | Specific activity (μmol $P_i$/min per mg protein) |
| Total homogenate | 7.63 | | 13.18 | |
| 600 × *g* supernatant | 4.63 | | 7.79 | |
| Microsomes I | 2.60 | 0.13 | 4.56 | 0.23 |
| Microsomes II | 1.58 | 0.11 | 2.46 | 0.17 |
| Microsomes III | 1.11 | 0.12 | 1.75 | 0.18 |
| Microsomes IV | 0.68 | 0.11 | 1.12 | 0.18 |

as does the first microsomal pellet. The RNA/protein ratio in this second "crop" of microsomes is essentially the same as that in the first. When collecting third and fourth "crops" of microsomes, additional amounts of material are obtained, although in diminishing quantities and with a slightly reduced RNA/protein ratio.

The distribution of two microsomal marker enzymes, NADPH-cytochrome *c* reductase and glucose-6-phosphatase, are shown in Table II. Calculating the recovery of microsomal marker enzymes from this table only about 35% of the total homogenate activity is found in microsomal fraction I. Adding to this figure the recovery in microsomes II, the total yield of microsomal marker enzymes is about 55%. The recovery figures calculated from Table II indicate that using a large number of repeated suspensions it would be possible to recover only 80—85% of the total enzyme activity of the liver endoplasmic reticulum in the microsomes, provided that the resuspension procedure does not damage cells not disrupted by the initial homogenization. The remaining 15—20% of the total enzyme activity is probably inside intact cells. Both enzymes in Table II show the same pattern, which is strong support for the conclusion that the figures represent the recovery of endoplasmic reticulum membranes. The specific activities of the enzymes in the different microsomal fractions are similar, indicating that a large degree of contamination does not occur. The purity of the preparations are illustrated in Table III, where the distributions of succinate cytochrome *c* reductase (mitochondria) [9], CMP-sialic acid transferase (Golgi apparatus) [10], β-glycerophosphatase (lysosomes) [9], and finally AMPase (predominantly plasma membranes) [11] are shown. The figures in the table indicate that contamination by mitochondria is relatively little in all of the microsomal fractions. The amount of mitochondrial protein contaminating the microsomes varies between 2.5 and 3% of the protein in the microsomal fractions. The amount of contaminating protein can be calculated from Table III by dividing the absolute activity of succinate cytochrome *c* reductase in the actual fraction with the specific activity of the enzyme mea-

TABLE III

DISTRIBUTION OF SOME MARKER ENZYMES IN MICROSOMAL FRACTIONS

Total homogenates were prepared as in Table II. Microsomes I—IV were prepared as in Table I. Mitochondria [15,16], Golgi fraction [12], lysosomes [17] and plasma membranes [18] were isolated using procedures described earlier. For the measurement of CMP-sialic acid transferase desialidated fetuin was used as an exogenous protein acceptor [10]. The values are the means of 5 experiments.

| | Succinate cytochrome *c* reductase | | CMP-sialyl transferase | | β-Glycerophosphatase | | AMPase | |
|---|---|---|---|---|---|---|---|---|
| | Abs. act. [a] | Spec. act. [b] | Abs. act. [c] | Spec. act. [d] | Abs. act. [e] | Spec. act. [f] | Abs. act. [e] | Spec. act. [f] |
| Total homogenate | 11.0 | 0.05 | 41.5 | 0.27 | 20.0 | 0.13 | 7.55 | 0.049 |
| Microsomes I | 0.13 | 0.007 | 10.30 | 0.50 | 1.28 | 0.07 | 1.20 | 0.06 |
| Microsomes II | 0.19 | 0.013 | 9.51 | 0.67 | 1.55 | 0.11 | 1.27 | 0.09 |
| Microsomes III | 0.10 | 0.010 | 6.62 | 0.69 | 0.47 | 0.05 | 1.18 | 0.13 |
| Microsomes IV | 0.05 | 0.008 | 4.45 | 0.73 | 0.28 | 0.05 | 1.15 | 0.21 |
| Mitochondria | 8.78 | 0.351 | | | | | | |
| Golgi fraction | | | 25.1 | 85.1 | | | | |
| Lysosomes | | | | | 1.87 | 1.12 | | |
| Plasma membranes | | | | | | | 0.31 | 0.73 |

[a] μmol cytochrome *c* reduced/min per g liver.
[b] μmol cytochrome *c* reduced/min per mg protein.
[c] pmol sialic acid transferred/min per g liver.
[d] pmol sialic acid transferred/min per mg protein.
[e] μmol inorganic phosphorous released/min per g liver.
[f] μmol inorganic phosphorous released/min per mg protein.

sured in the purified mitochondrial preparation. These values represent the maximal contamination possible, because the specific activity is based on protein from intact mitochondria.

Using CMP-sialic acid transferase as a marker of the Golgi system, it can be seen that almost 50% of the total Golgi membranes are recovered in microsomes I and II together, while the corresponding value for pooled microsomes I—IV is 74%. Microsomes III and IV have a higher degree of Golgi contaimination on a protein basis. On the other hand, the amount of Golgi protein in microsomes I and II is as low as 0.6—0.7% of the total microsomal protein. The value is calculated from Table III in the same manner as for mitochondria. Since Golgi vesicles, especially from animals which have been treated with alcohol, are filled with lipoprotein, they can easily be eliminated from the microsomes by flotation [12].

The β-glycerophosphatase activity values in Table III show that microsomes I are contaminated with lysosomes and microsomes II even more so. Further resuspensions and centrifugations result in only a limited increase in lysosomal contamination. Nonetheless, the specific activity of β-glycerophosphatase is low in pooled microsomes I and II. The amount of lysosomal protein in these microsomal fractions can be calculated from Table III to be 5—7% of the total.

AMPase activity is widely distributed among cellular organelles, including the endoplasmic reticulum and Golgi membranes [11], but is present at highest activity in plasma membranes. In Table III it can be seen that the specific activity of AMPase is drastically increased in microsomes III and is even higher in microsomes IV. Microsomes I and II together contain about 30% of the total AMPase activity in the cell, which, disregarding the fact that microsomes themselves contain AMPase, corresponds to contamination by 8—10% of plasma membrane protein. Taking into account the specific activity of AMPase in pure rough microsomal membranes (0.03 μmol phosphate released/min per mg of microsomal protein) [8] as a measure for the microsomal AMPase, contamination by plasma membranes can be calculated to amount to 5—6% of the total microsomal protein. This figure increases to 10—11% (100% increase) if all four microsomal fractions are pooled.

TABLE IV

CHEMICAL COMPOSITION OF MICROSOMAL SUBFRACTIONS

The subfractions were prepared as described earlier [7] and involved a cation-containing discontinuous sucrose gradient. RI, Ribosome-rich microsomes; RIII, ribosome-poor microsomes; Sm, smooth microsomes. Values are the means of 5 experiments.

| | Protein (mg/g liver) | Phospholipids | RNA | RNA / protein |
|---|---|---|---|---|
| Microsomes I | 19.8 | 6.2 | 2.30 | 0.12 |
| RI | 9.24 | 2.85 | 1.47 | 0.16 |
| RIII | 2.69 | 1.03 | 0.24 | 0.09 |
| Sm | 4.45 | 1.60 | 0.17 | 0.04 |
| Microsomes II | 14.1 | 4.6 | 1.6 | 0.11 |
| RI | 7.84 | 2.46 | 1.16 | 0.15 |
| RIII | 1.35 | 0.51 | 0.11 | 0.08 |
| Sm | 3.44 | 1.19 | 0.13 | 0.04 |

TABLE V

DISTRIBUTION OF SOME MARKER ENZYMES IN MICROSOMAL SUBFRACTIONS

Fractions prepared as in Table IV. Values are the means of 5 experiments.

| | Specific activity | | | | | |
|---|---|---|---|---|---|---|
| | NADPH-cytochrome *c* reductase (μmol cytochrome *c* reduced/min per mg protein) | Glucose-6-phosphatase (μmol $P_i$ released/min per mg protein) | Succinate-cytochrome *c* reductase (μmol cytochrome *c* reduced/min per mg protein) | CMP-sialyl transferase (pmol sialic acid transferred/min per mg protein) | β-glycerophosphatase (μmol $P_i$ released/min per mg protein) | AMPase (μmol $P_i$ released/min per mg protein) |
| Microsomes I | 0.13 | 0.23 | 0.007 | 0.50 | 0.07 | 0.06 |
| RI | 0.11 | 0.23 | 0.006 | 0.05 | 0.07 | 0.04 |
| RIII | 0.15 | 0.19 | 0.003 | 0.20 | 0.06 | 0.06 |
| Sm | 0.12 | 0.14 | 0.002 | 1.63 | 0.06 | 0.07 |
| Microsomes II | 0.11 | 0.17 | 0.013 | 0.67 | 0.11 | 0.09 |
| RI | 0.09 | 0.17 | 0.014 | 0.18 | 0.09 | 0.05 |
| RIII | 0.15 | 0.12 | 0.006 | 0.40 | 0.19 | 0.11 |
| Sm | 0.10 | 0.08 | 0.004 | 0.83 | 0.14 | 0.19 |

Summing up the information in this table, it is apparent that microsomes I and II together give a much higher recovery of endoplasmic reticulum with only a minimal increase in contaminating membranes. When microsomes III and IV are also pooled with the previous two fractions, however, the recovery only increases moderately and contamination increases greatly.

Subfractionation of microsomes I and II using cation-containing discontinuous sucrose gradients has also been performed. Table IV shows the chemical composition of microsomal subfractions prepared by using a cation-containing discontinuous sucrose gradient according to Eriksson [7]. The amount of ribosome-rich microsomes (RI), ribosome-poor microsomes (RIII) and smooth microsomes (Sm) is essentially the same in microsomes I and II.

Table V illustrates the distribution of some marker enzymes between RI, RIII and smooth microsomes. The distribution of microsomal marker enzymes, NADPH-cytochrome *c* reductase and glucose-6-phosphatase, is similar within microsomes I and II. In addition, it also appears that the contaminating activities increase in specific fractions; mitochondrial marker enzyme activity increases mainly in the ribosome-rich microsomes, while AMPase and β-glycerophosphatase are elevated in smooth microsomes. Most of the CMP-sialic acid transferase activity is found in smooth microsomes from microsomes I.

After treatment with drugs such as phenobarbital and methylcholanthrene some changes in the membrane amount and enzyme composition of microsomal fractions has been noticed. Table VI summarizes the distribution of membrane constituents in these cases. Looking at the phenobarbital-treated animals, a large increase in membrane amount and in the activity of NADPH-cytochrome *c* reductase activity, but not in AMPase activity can be seen. The

TABLE VI

DISTRIBUTION OF SOME MEMBRANE CONSTITUENTS IN MICROSOMAL FRACTIONS FROM THE LIVERS OF PHENOBARBITAL- AND METHYLCHOLANTHRENE-TREATED RATS

Rats were treated with phenobarbital (8 mg/100 body weight per day) for three days, or with 3-methylcholanthrene (2 mg/100 g) 5 and 3 days before sacrifice. The values are the means of 4 experiments.

| | Protein | Phospholipids | RNA | NADPH-cytochrome *c* reductase | | AMPase | |
|---|---|---|---|---|---|---|---|
| | | | | Absolute activity [a] | Specific activity [b] | Absolute activity [c] | Specific activity [d] |
| Control | | | | | | | |
| Microsomes I | 19.8 | 6.2 | 2.3 | 2.60 | 0.13 | 1.20 | 0.07 |
| Microsomes II | 14.1 | 4.6 | 1.6 | 1.58 | 0.11 | 1.27 | 0.08 |
| Phenobarbital | | | | | | | |
| Microsomes I | 28.0 | 9.3 | 2.8 | 5.96 | 0.21 | 1.00 | 0.04 |
| Microsomes II | 20.1 | 6.7 | 1.9 | 4.41 | 0.22 | 0.91 | 0.05 |
| Methylcholanthrene | | | | | | | |
| Microsomes I | 21.0 | 6.5 | 2.1 | 2.42 | 0.12 | 1.04 | 0.05 |
| Microsomes II | 19.3 | 5.9 | 1.5 | 2.02 | 0.11 | 1.18 | 0.06 |

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

distributions between microsomes I and II are quite comparable with those of the control microsomes. In the case of methylcholanthrene treatment a 30% increase in the membrane amount can be seen. This increase can scarcely be seen by just studying microsomes I, but is clearly revealed by studying microsomes II. On the other hand the specific activities of the enzymes studied are not different.

## Discussion

Isolation of a microsomal fraction with a relatively high recovery of endoplasmic reticulum fragments is important from several points of view. Various types of investigations require large amounts of microsomal material from the same liver. What is more important is obtaining a representative fraction and avoiding loss of a specific portion of the endoplasmic reticulum. Since the total microsomal fraction is composed of a number of subfractions with heterogeneous enzymatic patterns, it may well be that the microsomes in the 10000 $\times g$ pellet represent a specific part of the endoplasmic reticulum system. This problem is accentuated after membrane induction with drugs, where the type, the number and the properties of the vesicles are changed.

This study demonstrates that a mild resuspension and centrifugation of the first 10000 $\times g$ pellet may be performed without causing breakage of the various organelles and, consequently, without increasing contamination. This statement is valid only when homogenization of the liver is performed in a relatively mild manner and most of the cytoplasmic organelles are intact. Mild homogenization is also necessary to avoid the breakage of nonparenchymal cells, which have a structural and functional organization different from that of the major part of the liver cells. Furthermore, it is improbable that the repeated resuspension could cause contamination of the parenchymal cell membrane fraction with endoplasmic reticulum from the sinusoidal lining cells by causing breakage of nonparenchymal cells. In a recent investigation, Blouin et al. [14] concluded that the non-parenchymal cells constitute only 6% of the parenchymal volume and also that their endoplasmic reticulum represents no more than 6% of that of the total liver.

One additional resuspension of the 10000 $\times g$ pellet takes only a little extra time and the reward is substantial since the recovery is almost doubled or, in the case of methylcholanthrene-treated rats, doubled. In particular cases where the highest possible recovery is required one can collect additional crops and accept the increased contamination.

The specific activity of microsomal enzyme activities in all those fractions which are recovered by additional resuspensions of low speed sediments are very similar to those found in the membranes from the first 10000 $\times g$ supernatant. Consequently, the microsomes I are representative membranes of the liver endoplasmic reticulum and analyses of this fraction will give a true picture of the state of this membrane system. On the other hand, it cannot be excluded that under certain pathological conditions vesicles with high sedimentation velocity and changed structure and function may sediment at a low centrifugal force, and in such a case pooling of microsomes II and microsomes I will be a necessity.

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