MICROMETHOD FOR THE PREPARATION OF A MICROSOMAL FRACTION FROM RAT AND HUMAN LIVER BY DIFFERENTIAL SEDIMENTATION

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Received November 11,1974

SUMMARY

Microsomes were prepared from small amounts of rat liver (20 mg). The purity of the fraction was demonstrated by the determination of marker enzymes for the mitochondrial (glutamate dehydrogenase), lysosomal (acid phosphatase), peroxisomal (uricase), and microsomal (glucose-6-phosphatase) cell organelles. A modified ultra-micromethod was used for protein determination. With this procedure microsomes from needle biopsy samples of human liver have been isolated, and their total amount is calculated by using cytochrome P-450 as marker enzyme.

INTRODUCTION

Data about the normal activity of microsomal enzymes in human liver are almost lacking. Liver samples removed during abdominal surgery should be regarded as a selected material (1). Besides, the influence of anaesthetics on drug metabolizing liver enzymes should be avoided. In a previous paper (2) the drug metabolizing enzyme system has been explored in total homogenate of needle biopsy material from human liver. Because of latent interferences by enzymes located in other cell organelles, coenzymes, substrates, and a high protein concentration, with the measurement of microsomal enzymes in homogenate, this report presents a procedure for preparing microsomes from amounts of rat liver tissue which would be also available from needle biopsy material of human liver.

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MATERIALS AND METHODS

Cell fractionation: Pieces of 15-20 mg, either from livers of 120-200 g male Wistar rats or from needle biopsy material of human liver, have been homogenized with SVT buffer (2). The homogenate was then finally diluted to about 1 : 50 (15-20 mg liver/ml).

Preparation of microsomes: After having removed some of the 1 : 50 dilution for the determination of enzyme activities and the protein content in the total homogenate, the remaining volume of 1 ml was first centrifuged in micro-tubes. Special plastic adaptors for the SS 34 rotor were used. After 10 min at 40 x g, 10 min at 550 x g, and 10 min at 950 x g, the supernatant was separated from the pellet and transferred into a 2 ml nitro cellulose tube. The pellet was washed once with buffer and again centrifuged at the same speed as before. The remaining pellet is called NMLP-fraction (nuclear, mitochondrial, lysosomal and peroxisomal fraction). The second and the first 900 x g supernatant were mixed in the nitro cellulose tube and centrifuged for 45 min in the Spinco L (0° C) with the 50 rotor at 106 000 x g. The pellet is called R-fraction (microsomal fraction), the final supernatant, S-fraction. The pellet of the NMLP- and R-fraction was resuspended and stored at 4° C until use. Aliquots of homogenate and tissue fractions were transferred to small tubes (1-5 ml), frozen immediately and lyophilized at -40° C. Following lyophilization, samples were sealed and stored at -20° C for further studies. All dilution steps and the removal of the supernatants were corrected by weighing out the volumes.

Enzyme assays: Glucose-6-phosphatase (E.C. 3.1.3.9). This enzyme has proven to be strongly bound to microsomal membranes in rat hepatocytes (3, 4). The incubation was carried out, according to Harper (5), with cacodylate buffer, and the inorganic phosphate was estimated by the method of Taussky and Shorr (6).

Glutamate dehydrogenase (E.C. 1.4.1.3). The activity of this mitochondrial marker enzyme (7) was measured in freeze-dried tissue fractions. A method, according to Mattenheimer (8) (direct optical test), with 1 mM ADP (7) in the incubation mixture was used.

Acid phosphatase (E.C. 3.1.3.2). A working hypothesis about the origin of acid phosphatases will be found by Ide and Fishman (9). An extralysosomal location of β-glycerophosphatase is not to be expected. The activity was assayed by the method of Appelmans et al. (10) and Wattiaux et al. (11) in freeze-dried tissue fractions or homogenate with β-glycerophosphate as substrate.

Uricase (E.C. 1.7.3.3). This enzyme is firmly attached to an unsoluble com-

partment of hepatic microbodies (12). Its activity was measured with the direct spectrophotometrical test, according to Mahler et al. (13, 14, 15), in freeze-dried homogenate and cell fractions. The reaction mixture with the enzyme (cell fraction) was preincubated for 4 min, and the reaction started by adding the uric acid solution.

Cytochrome P-450. The cytochrome difference spectrum was recorded with an Aminco DW-2 UV-VIS spectrophotometer (2).

About 10-30 µg microsomal protein were needed for one measurement of each enzyme assay. Only for cytochrome P-450 the required amount, 0.25 mg protein for one measurement, was about ten times higher. The incubation volumes ranged between 15 and 40 µl. In direct spectrophotometrical determinations the cuvette volume was 200-300 µl.

Protein determination: The protein of all cell fractions and homogenates was determined with Folin's phenol reagent (16) after precipitation by TCA, as it is described in detail elsewhere (17).

RESULTS AND DISCUSSION +)

Because of the lower viscosity of the highly diluted suspension, a higher aggregation of the different particles, and of the fact that the height of the fluid column in micro-tubes, measured as horizontal projection, was only one third of that in macro-tubes, the g-number for sedimentation of the NMLP-fraction had to be reduced considerably. The marker enzymes were selected by postulating that the enzyme chosen is located exclusively in one type of cell organelles. Based on the activity of the reference enzyme glucose-6-phosphatase, the microsomal fraction contains 54 per cent of all microsomes present in the liver. It is contaminated with 3.2 per cent of mitochondria, as the GLDH activity indicates (Tab. 1), as well as with 35.7 per cent of lysosomes and 44 per cent of peroxisomes. 14.1 per cent of the total protein were found in the microsomal fraction. Since only 54 per cent of all the microsomes could be isolated, the total microsomal protein amounts to 26.1 per cent of the cell protein, corresponding to 49.6 mg microsomal protein per g of rat liver. This value lies in the lower

+) For intracellular distribution patterns of enzymes in rat liver tissue see also De Duve et al., 1955 (19).

TABLE 1. Distribution of reference enzymes in 3 cell fractions prepared from 20 mg of rat liver

Enzyme	Number of experiments (animals)	Absolute values of total amounts	Per cent of recovered amount				Recovery
			NMLP+R+S	NMLP	R	S	
Protein	14	190.2 ± 12.6	100	43.1 ± 3.9	14.1 ± 1.0	42.8 ± 3.8	96.2 ± 6.4
Glucose-6-phosphatase	12	15.82 ± 2.45	100	45.4 ± 7.7	54.0 ± 9.8	0.8 ± 0.2	89.0 ± 13.8
Glutamate dehydrogenase	14	200.3 ± 30.9	100	92.2 ± 13.8	3.2 ± 0.8	4.6 ± 2.2	100.7 ± 15.5
Acid phosphatase	14	3.17 ± 0.37	100	54.9 ± 11.4	35.7 ± 6.0	9.5 ± 2.5	96.9 ± 11.3
Uricase	7	1.41 ± 0.21	100	56.0 ± 8.5	44.0 ± 8.5	< 1.1	92.8 ± 13.8

Total amounts (sum of the fractions) are given in enzyme units and in mg of protein per 1 g of liver. Recoveries are expressed as per cent of total amounts compared with findings in total homogenate.

NMLP = nuclear, mitochondrial, lysosomal and peroxisomal fraction;
R = microsomal fraction; S = final supernatant.

The numbers state the determinations of unpooled liver tissue from different animals.

TABLE 2. Cytochrome P-450 content in the liver of male Wistar rats (body weight 190-230 g) and of patients determined with micromethods

	n	n moles/mg Microsomal protein [1]	n	n moles/g Liver wet weight [2]	mg Microsomal protein
Rat	6	0.79 ± 0.09	-	-	-
Man	3	0.36 ± 0.04	20	10.8 ± 2.6	30.0

The numbers (n) state the determinations of unpooled liver tissue from different animals and men.

1) - determined in microsomes 2) - determined in homogenate

range found by Amar-Costesec et al. (18).

According to the best values in literature, peroxisomes and lysosomes contain 1.4 per cent (De Duve et al., 1966 (20)) and 0.65 per cent (Baudhuin (21)), respectively, of the total liver cell protein. Using these values and taking into account that 3.2 per cent of the mitochondria, containing about 0.9 per cent (= 1.8 mg) of the cell protein, are present in the microsomal fraction, we calculated its contamination with protein from other cell granules as being about 12 per cent. This seems to be acceptable. When microsomes were prepared by the original macromethod, a contamination by mitochondria alone was shown by other investigators to be 6 per cent (rat) and 12 per cent (human), respectively (Ackermann et al. (1)). A criterion for the purity of the microsomal fraction is the relative specific activity of glucose-6-phosphatase (percentage of activity / percentage of protein), which is calculated to 3.53 (54.0/14.1). Similar values have been found by other authors (De Duve et al., 1955 (19); Ackermann et al. (1); Barrow et al. (22)).

Microsomes isolated with this procedure contained 0.79 n moles cytochrome P-450 per 1 mg protein (this enzyme is also located exclusively in microsomes).

The described micro-procedure seems to be suitable for preparing a

microsomal fraction from human liver. By using this micromethod we got values for the cytochrome P-450 content in the microsomal fraction of rat and human liver, as they are compared in Table 2. The cytochrome P-450 content in human endoplasmic reticulum reaches only 40-50 per cent of the values found in rat liver microsomes. By comparing the amount of cytochrome P-450 in the isolated microsomal fraction with that in liver homogenate +) we are able to calculate the total amount of microsomes in human liver cells to about 30 mg microsomal protein per g liver, indicating a smaller amount than can be found in rat liver. More precise studies about this subject are progressing in this laboratory.

ACKNOWLEDGEMENTS

This research was supported by a grant from the Liver Research Fund, Presbyterian-St. Luke's Hospital, Chicago, and by a grant-in-aid from the German Research Association (Deutsche Forschungsgemeinschaft).

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+) The latter we had previously determined in 20 needle biopsy samples of normal human liver (2), giving a value of 10.8 ± 2.6 n moles per g liver.

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