ISOLATION AND PROPERTIES OF ROUGH AND SMOOTH VESICLES FROM RAT LIVER

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The endoplasmic reticulum of the liver cell appears in thin sections as a system of channels, composed of membranes, the majority of which have ribonucleoprotein (RNP) particles attached to their outer surfaces. The microsomal fraction, isolated after homogenization and differential centrifugation, contains free RNP[1] particles and two kinds of vesicles: rough, with RNP particles attached to the outer surface, and smooth, which lack these particles (1). Recently many efforts have been made to separate these two structures and to characterize them (2–5). Long centrifugation times were necessary to obtain relatively pure fractions. In the following, a rapid and simple method for isolation of rough and smooth vesicles from rat liver homogenate will be described together with some of the main enzymic characteristics of these structures.

MATERIALS AND METHODS

All fractionations were performed with a commercial Christ ultracentrifuge, model Omega. An angle-head rotor was used which can take eight plastic centrifuge tubes, each with a capacity of 11.5 ml. The tube angle with the rotor axis is 20°. At the maximal speed, 60,000 RPM, the centrifugal force is 250,000 *g*, as calculated for the middle of the tube.

In all experiments, rats of the inbred, homozygote strain R/StDa (strain R, subline Stockholm-Dallner) were used. All animals fasted overnight. The livers were homogenized in enough 0.30 M sucrose to give a 25 per cent homogenate, which was centrifuged at 20,000 *g* for 30 minutes to eliminate cell debris, nuclei, and mitochondria. The supernatant, containing most of the microsomes, was decanted. To 9.9 ml of this supernatant, 0.1 ml of 1 M $MgCl_2$ was added. This will be referred to as "supernatant A."

To obtain the total microsomal fraction, 7 ml of the supernatant A was diluted with 4.5 ml of a 0.3 M sucrose–0.01 M $MgCl_2$ solution and centrifuged at 250,000 *g* for 60 minutes. The pellet was rinsed three times and resuspended by homogenization in 0.3 M sucrose.

For separation of smooth (*a*) and rough (*b*) vesicles, 7 ml of the supernatant A was layered over 4.5 ml of a 1.5 M sucrose–0.01 M $MgCl_2$ solution. After centrifugation at 250,000 *g* for 30 minutes, a tight, light brown pellet was obtained and a red fluffy layer was found at the boundary of the two phases.

(*a*) The upper phase (7 ml) including the fluffy layer was aspirated, diluted with 4.5 ml 0.3 M sucrose, and recentrifugated at 250,000 *g* for 60 minutes. The pellet obtained, called "subfraction I," was rinsed three times and used either for investigation by electron microscopy or resuspended by homogenization in 0.3 M sucrose for biochemical analysis.

(*b*) The lower 1.5 M sucrose layer was discarded and the tight bottom pellet, labeled "subfraction II," was rinsed three times. This pellet was treated in the same way as subfraction I.

In order to maintain the structural and func-

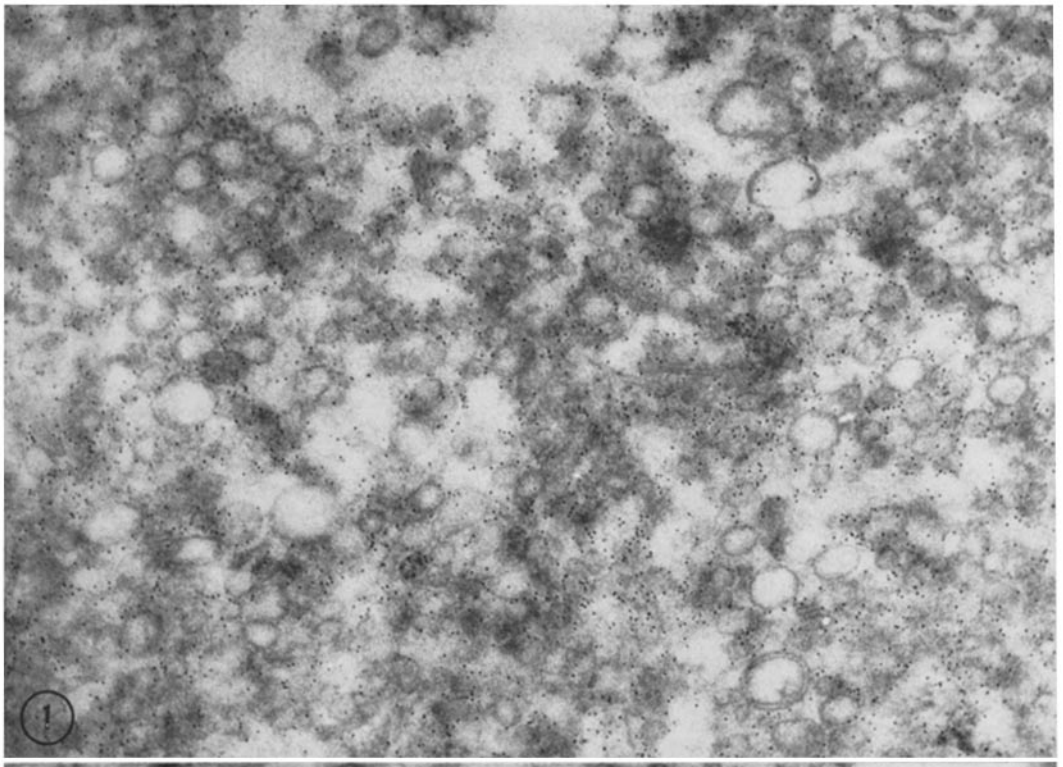
[1] Abbreviations used are: RNP: ribonucleoprotein; DPNH and TPNH: reduced diphosphopyridine and triphosphopyridine nucleotides; DIC: dicoumarol; DOC: deoxycholate; ATP: adenosinetriphosphate; ADP: adenosinediphosphate; IDP: inosinediphosphate.

FIGURE 1

Subfraction I. Smooth vesicles and numerous particles presumably ferritin. × 60,000.

FIGURE 2

Subfraction II. Essentially rough vesicles and free RNP particles. Some of the smallest vesicles may be of the smooth type. × 60,000.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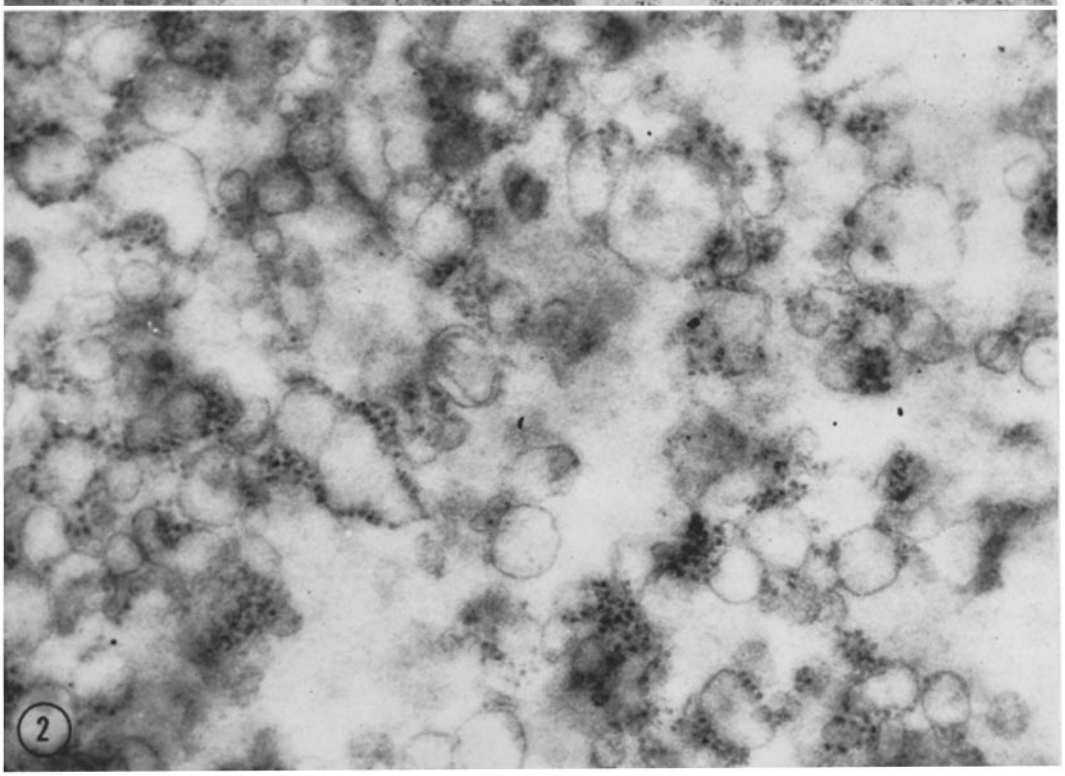
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tional integrity of the free RNP particles, $MgCl_2$ was used during the procedure (6). This caused these particles to sediment along with the rough vesicles.

All pellets were so hard that they could easily be removed from the tubes with a small spatula. For investigation at the ultrastructural level they were placed in 1 per cent buffered OsO_4 (pH 7.2) (7) and cut into small pieces. After fixation for 1 hour, the specimens were embedded in Epon (8). Sections were cut at different levels of the pellets and examined in a Siemens Elmiskop I at magnifications ranging from 18,000 to 30,000.

Protein was measured by the method of Lowry *et al.* (9). Ribonucleic acid was estimated according to Ceriotti (10). Lipids were extracted (11) and the phosphorus content estimated (12). The value obtained was multiplied by 25 to get the total amount of phospholipids (13). Enzyme assays were performed according to previously described methods (13–15).

The preparatory procedure was repeated many times and in all cases the biochemical and morphological data were similar. The results from one such experiment are recorded below.

RESULTS AND COMMENTS

In all experiments, electron microscopical investigation showed striking similarity of pellets from the same subfraction at all levels examined. Subfraction I contained smooth vesicles and small particles, presumably ferritin (Fig. 1). Rough vesicles or free RNP particles could not be detected. The sections of subfraction II showed predominantly larger vesicles with RNP particles attached to the outer surface of the membranes and many free RNP particles between the vesicles (Fig. 2). It cannot be excluded that some of the smaller vesicles are of the smooth type.

The results of biochemical analysis (Table I) indicate a low RNA/protein ratio in subfraction I and a high ratio in subfraction II. This is in agreement with the values previously presented by other authors (1, 2) for smooth and rough

TABLE I

Distribution of Protein, RNA, and Phospholipids in the Total Microsomal Fraction and Subfractions

Fractions	Protein mg/gm liver	RNA mg/gm liver	Phospholipids mg/gm liver	RNA / Protein	Phospholipids / Protein
Total microsomal fraction	32.6	6.72	13.6	0.21	0.42
Subfraction I (smooth vesicles)	6.6	0.31	3.6	0.05	0.55
Subfraction II (rough vesicles + free RNP particles)	23.0	6.08	8.1	0.26	0.35

TABLE II

Diaphorase and Cytochrome c Reductase Activities in Total Microsomal Fraction and Subfractions

The diaphorase and cytochrome *c* reductase activities were measured according to Ernster *et al.* (13, 15). Diaphorase activities of all test systems were measured in the presence of 3 mg per ml of Tween-20. All values are expressed in terms of μmoles DPNH or TPNH oxidized per minute.

	Diaphorase activity								Cytochrome *c* reductase activity			
	DPNH		DPNH + DIC		TPNH		TPNH + DIC		DPNH		TPNH	
Fractions	per gm liver	per mg protein	per gm liver	per mg protein	per gm liver	per mg protein	per gm liver	per mg protein	per gm liver	per mg protein	per gm liver	per mg protein
Total microsomal fraction	13.70	0.42	12.70	0.39	2.29	0.07	1.63	0.05	33.60	1.03	1.30	0.04
Subfraction I	0.66	0.10	0.53	0.08	0.26	0.04	0.07	0.01	1.92	0.29	0.07	0.01
Subfraction II	12.70	0.55	13.30	0.58	2.53	0.11	2.53	0.11	31.70	1.38	1.15	0.05

membranes. The same is true for the phospholipid/protein ratio (1, 2).

An interesting finding in the study of the electron-transporting enzymes was the total lack of DT diaphorase activity in subfraction II (Table II). DT diaphorase, which was purified by Ernster *et al.* (16, 15), reacts at equal rates with both DPNH and TPNH and is highly sensitive to dicoumarol. Dicoumarol-sensitive DPNH and TPNH (non-specific) activity was found exclusively in subfraction I. On the contrary, more than 90 per cent of the specific DPNH- and TPNH-oxidizing activities were found in subfraction II. As electron acceptor, 2,6-dichlorophenolindophenol (diaphorase activities) or cytochrome *c* (cytochrome *c* reductase activities) was used. These enzyme activities, calculated on a protein basis, were also much higher in subfraction II. Repeated washings of subfraction I did not diminish the DT diaphorase activity, which excluded a possible absorption of soluble cytoplasmic DT diaphorase by smooth vesicles. When this fraction was treated by a Super Turrax blender (17), the enzyme could be extracted quantitatively. This property was earlier found to be very characteristic of the microsomal DT diaphorase and led to the conclusion that this enzyme is possibly bound to the membrane structure only by mechanical forces, in contrast to the other electron-transferring enzymes which are probably chemically bound (18, 19). The different electron-transferring enzymes were identified furthermore by their reactivity with quinones, tetrazolium dyes, and ferricyanide (20).

Recently Fouts reported (4) a higher "TPNH oxidase" in smooth-surfaced vesicles from rabbit liver in comparison with rough vesicles. Using our method, we also performed subfractionations of the rabbit liver microsomal fraction, and a concentration of TPNH diaphorase and cytochrome *c* reductase activities was found in subfraction I, which contained smooth vesicles. A comparison of the enzymic pattern of the two microsomal subfractions in different species will be published subsequently. A direct comparison between our results and those of Chauveau, Moulé, Roullier, and Schneebeli (5), and Ernster, Siekevitz, and Palade (13) cannot be made since their fractionation procedures differ greatly from ours.

TABLE III

Glucose-6-phosphatase, Nucleoside Tri- and Diphosphatase Activities in Total Microsomal Fraction and Subfractions

Glucose-6-phosphatase, nucleoside tri- and diphosphatase activities were measured according to Ernster and Jones (14). DOC concentration was 0.1 per cent.

Additions	Total microsomal fraction μmoles P_i/20 min.		Subfraction I μmoles P_i/20 min.		Subfraction II μmoles P_i/20 min.	
	per gm liver	per mg protein	per gm liver	per mg protein	per gm liver	per mg protein
Glucose-6-phosphate	96.4	2.96	4.3	0.65	89.0	3.87
ATP	90.6	2.78	14.3	2.17	71.1	3.09
ADP	51.8	1.59	7.3	1.10	43.9	1.91
ADP + DOC	32.3	0.99	3.5	0.53	28.5	1.24
IDP	98.5	3.02	17.4	2.63	95.0	4.13
IDP + DOC	188.7	5.79	26.7	4.03	174.7	7.58

Glucose-6-phosphatase activity was present almost exclusively in subfraction II (Table III). This finding is of special interest, as this enzyme has a well defined function in cell metabolism (21). Nucleoside diphosphatase activities, with and without addition of DOC, and nucleoside triphosphatase activity of the two subfractions were also investigated. The results are similar to those described by Ernster and Jones (14) for total liver microsomes. These enzyme activities were somewhat higher in subfraction II, although the specific activity in this fraction was never greater than twice that of subfraction I.

The conclusions are summarized in the following points: 1. The preparation method described is rapid and simple and permits a separation of smooth and rough vesicles from the microsomal fraction of rat liver. 2. The fraction consisting of

smooth vesicles contains all the microsomal DT diaphorase. This finding provides a suitable test for this fraction, as measurement of DT diaphorase activity is very simple. 3. The majority of specific pyridine nucleotide-oxidizing enzymes is located in the fraction containing mainly the rough vesicles. 4. Glucose-6-phosphatase activity is almost exclusively limited to the latter fraction. 5. These results indicate that the two kinds of microsomal vesicles from rat liver differ not only ultrastructurally but also functionally, and should perhaps be regarded as two different organelles.

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