ZONAL CENTRIFUGATION OF MICROSOMES FROM RAT LIVER: RESOLUTION OF ROUGH- AND SMOOTH-SURFACED MEMBRANES

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The microsomal fraction of rat liver consists of free ribosomes and smooth- and rough-surfaced vesicles that are derived from fragmentation of the endoplasmic reticulum (1). Two methods, based on differences in density and sedimentation rate, have been used to subfractionate microsomes from rat liver into predominantly smooth- and rough-surfaced fractions (2,3). However, both methods have been hampered by the small capacities of commercially available high-speed centrifuge rotors, difficulties in maintaining gradient stability during acceleration and deceleration, and problems in loading the gradient and the sample. The development of the zonal ultracentrifuge system (4) has largely overcome these difficulties. This communication reports our results on the separation of different elements of the microsomal fraction in a B-XXIX zonal rotor.

METHODS

Normal and phenobarbital-treated (5) female rats (Charles River, CD^{R} Strain), weighing 150-250 g, were fasted 20 hr and

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then decapitated. The livers were excised and placed at once in cold 0.25 M sucrose; they were washed clean of blood by rinsing with cold sucrose, weighed, and cut into small pieces with scissors. The liver was homogenized with three up-and-down strokes in a Potter-Elvehjem glass-Teflon homogenizer containing 4 ml of 0.25 M sucrose/g tissue. Large fragments of connective tissue were removed by filtration through two layers of cheese-cloth. The homogenate was centrifuged at 10,000 × *g* for 10 min to eliminate cell debris, nuclei, and mitochondria; the micro-somes remained in the supernatant. This "10,000 × *g*" supernatant was decanted and 0.15 ml of 1 M CsCl was added to each 9.85 ml, giving a final concentration of 15 mM Cs^+. Thirty milliliters of this mixture, containing the equivalent of 8-9 g tissue based on original wet weight, was fractionated in a Spinco ultracentrifuge (Model L2-65B) equipped with a B-XXIX rotor at 25,000 rpm for 2 hr (equivalent to $5 \times 10^{10}\ \omega^2 t$). Anderson and coworkers (6) have described the characteristics of the B-XXIX rotor in detail. A 15 mM-CsCl solution (1200 ml) containing from 10% to 55% (w/w) sucrose was used as the gradient. All operations, including introduction of the sample layer and buffer overlayer (200 ml 0.005 M Tris-HCl, pH 7.5) and the recovery of the gradient containing the separated particles, were accomplished while the rotor was rotating at 3500 rpm. The gradient samples were continuously monitored with a Gilford recording spectrophotometer at 280 mμ.

Thirty-six fractions of 40 ml each were collected by displacing the gradient with 55% sucrose. The concentration of sucrose within the isolated fractions was analyzed with a Bausch and Lomb refractometer. Protein was measured by the method of Lowry et al (7); serum albumin was used as a standard for these

measurements. The microsomal lipids were extracted by the procedure of Bligh and Dyer (8) and the amount of phosphorus was determined according to Harris and Popat (9). All phosphorus values were multiplied by 25 to obtain the total amount of phospholipid. RNA was extracted and determined according to the procedure described by Schneider (10); hydrolyzed yeast RNA was used as standard. The marker enzymes for the microsomal fraction, NADPH diaphorase and NADH-cytochrome c reductase, were assayed as described by Dallner (2).

For electron microscopy, the fractions isolated by zonal centrifugation were diluted with 0.005 M Tris-HCl buffer (pH 7.5) and then centrifuged at 105,000 × *g* for 60 min. Representative pellets of these fractions were fixed 15 to 45 min in 2.5% gluteraldehyde buffered with 0.1 M sodium cacodylate (pH 7.2), rinsed three times in buffer alone, postfixed 1 hr in cacodylate buffered 1% OsO_4 (pH 7.2) and embedded in Epon 812 (11). Ultrathin sections were double stained with saturated aqueous uranyl acetate and lead citrate before examination in the electron microscope.

RESULTS AND DISCUSSION

The results of a typical experiment in which the rat liver postmitochondrial supernatant was subjected to zonal centrifugation in a B-XXIX rotor for 2 hr at 25,000 rpm are shown in Fig. 1. This procedure separated the original supernatant into three fractions as indicated by the UV absorption peaks at 280 mμ. Electron microscopy disclosed that Fraction II contained mostly smooth-surfaced vesicles with some contamination of free ribosomes (Fig. 2), whereas Fraction III consisted mainly of rough-surfaced vesicles (Fig. 3). The majority of free ribosomes were found in the higher density region of the soluble phase (Fraction I). The

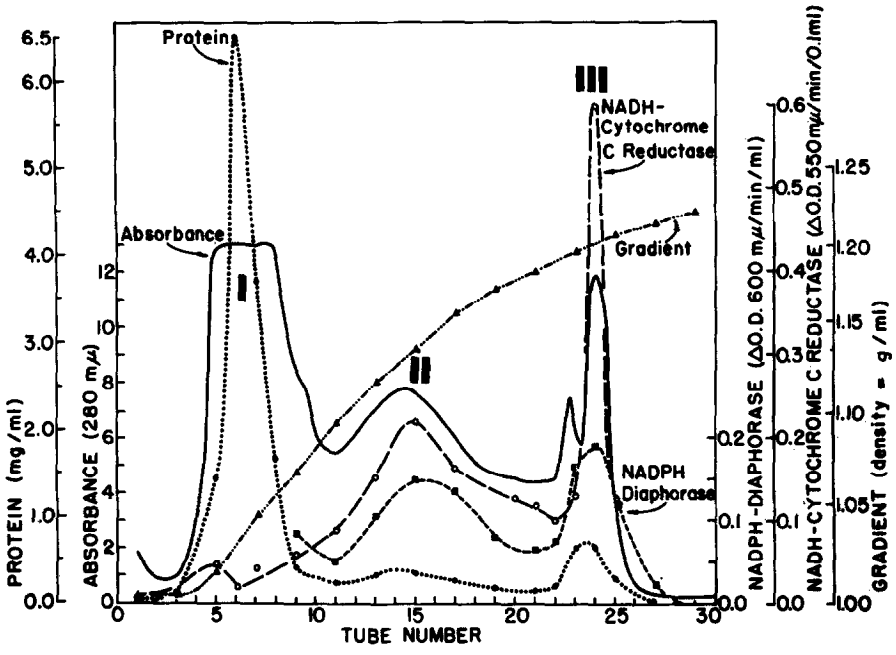

Figure 1. Zonal profile of absorbance, density, protein, NADH-cytochrome c reductase and NADPH diaphorase from rat liver postmitochondrial fraction. Centrifugation was to 5×10^{10} $\omega^2 t$ (25,000 rpm, 2 hr, B-XXIX rotor).

peaks consisting of smooth- and rough-surfaced vesicles were further characterized by NADH-cytochrome c reductase and NADPH diaphorase activities. The protein, phospholipid, and RNA contents of the two submicrosomal fractions are listed in Table 1. Protein was equally distributed in the two subfractions, but phospholipids were higher in the fraction containing smooth-surfaced vesicles. The high concentration of RNA in this fraction may be explained by the contamination with free ribosomes.

The zonal profile of the postmitochondrial fraction of livers from phenobarbital-treated rats is shown in Fig. 4. The protein ratio between smooth- and rough-surfaced membrane fractions was 1.46, whereas the ratio was 1.04 for the microsomal fractions derived from normal liver. Phenobarbital treatment causes the

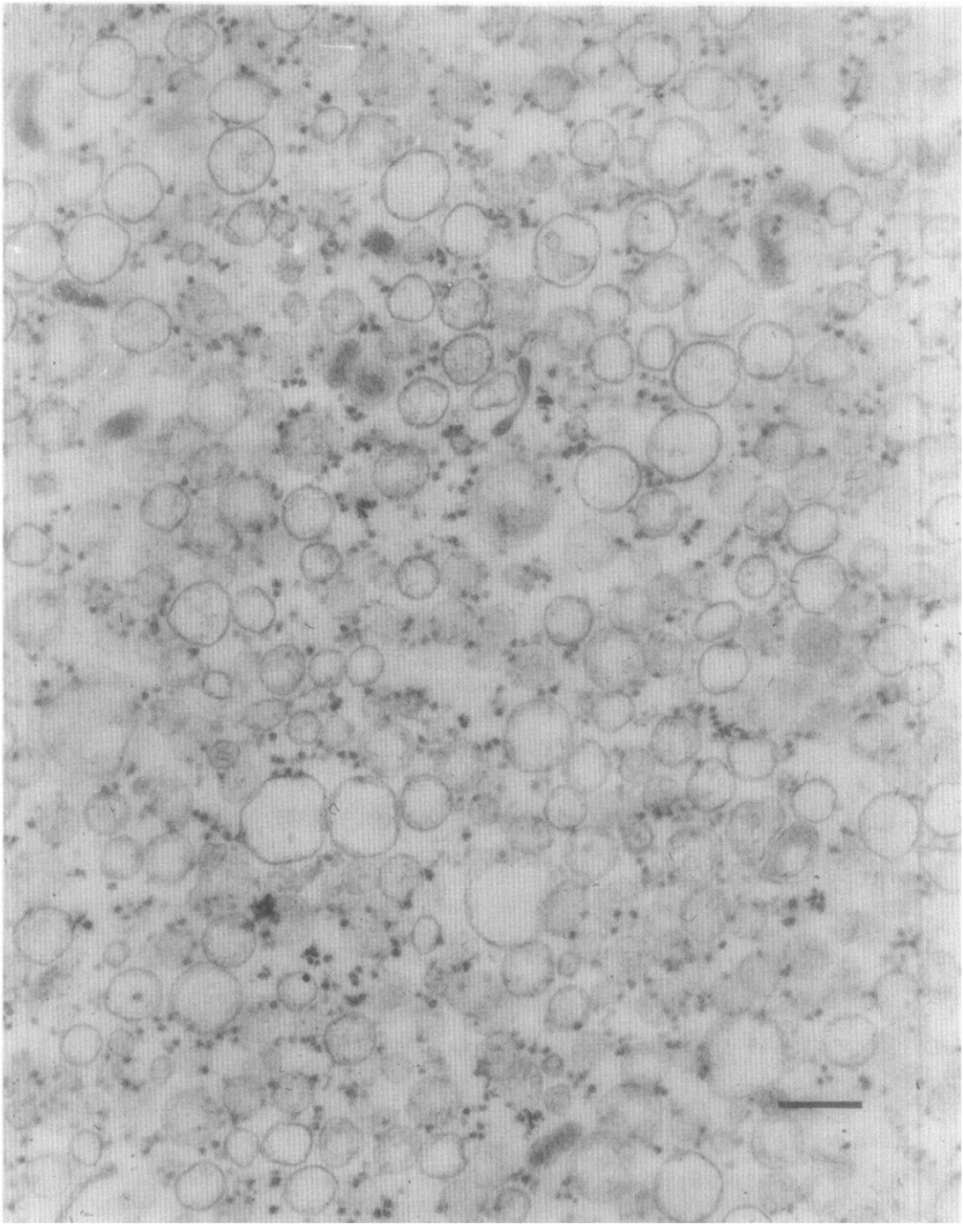

Figure 2. Electron micrograph of pellet obtained from gradient fraction 15 of the run shown in Fig. 1. All vesicles are of smooth type with some contamination by free ribosomes. Marker bar represents 0.25 μm.

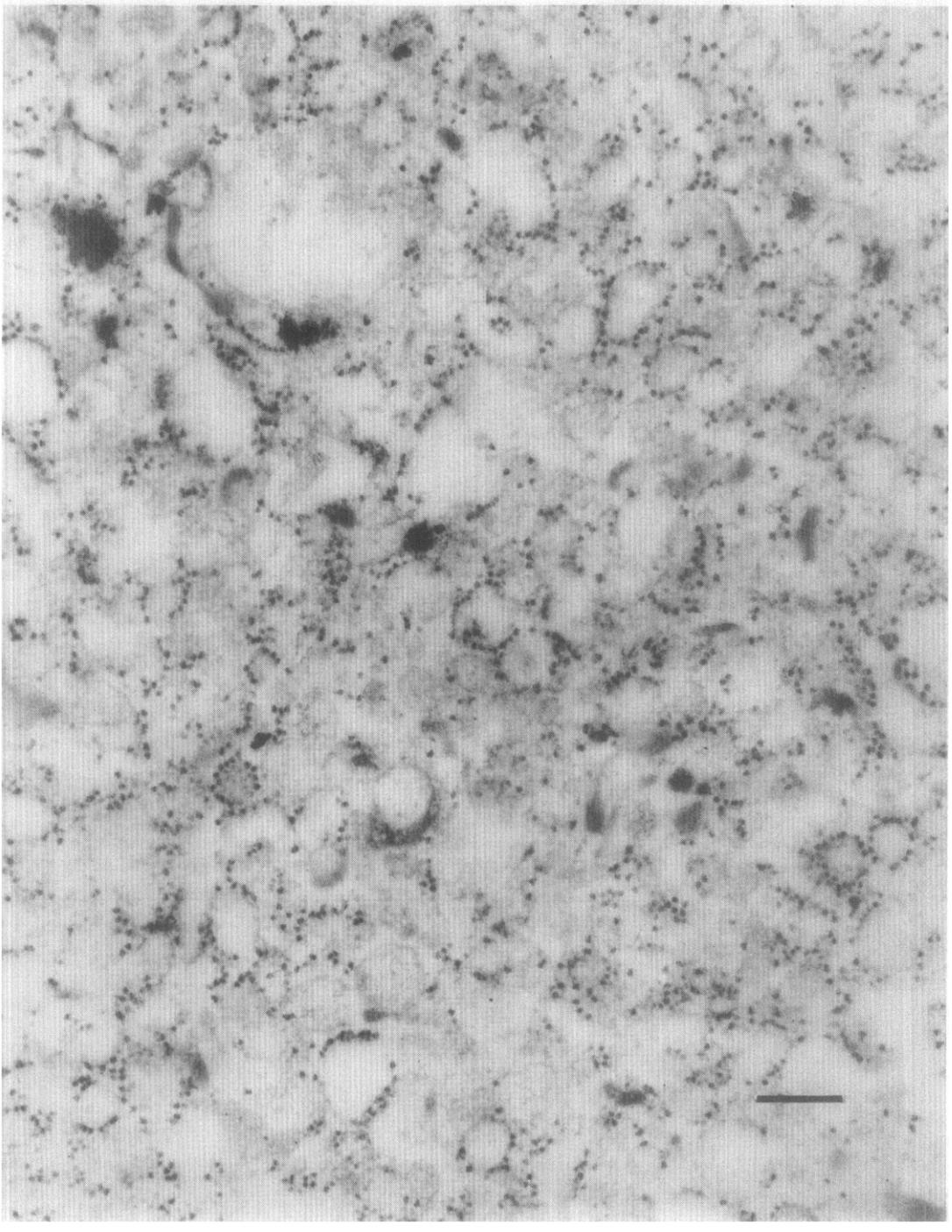

Figure 3. Electron micrograph of pellet obtained from gradient fraction 24 of the run shown in Fig. 1. All vesicles are of rough type. Marker bar represents 0.25 μm.

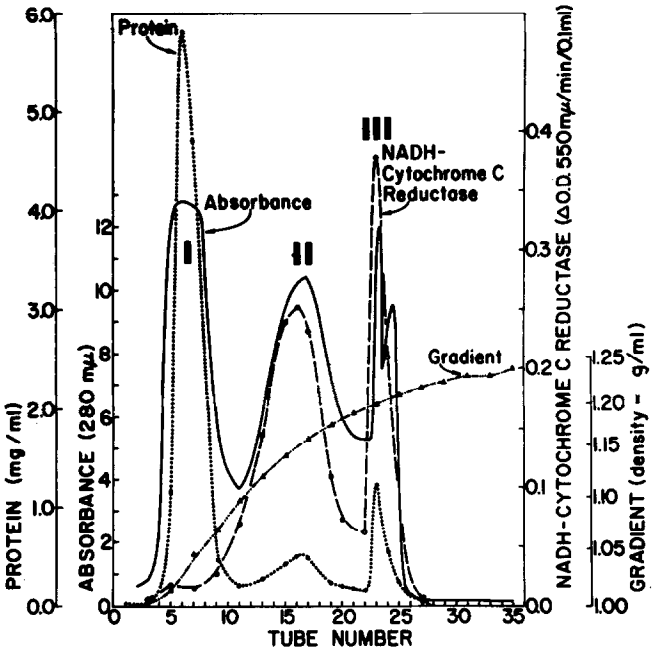

Figure 4. Zonal profile of absorbance, density, protein, and NADH-cytochrome c reductase obtained for postmitochondrial fraction of liver from phenobarbital-treated rat. The experimental animals received daily intraperitoneal injections of 9 mg of sodium phenobarbital/100 g body weight for 2 days. Other conditions used are described in the text.

TABLE 1

PROTEIN, PHOSPHOLIPID, AND RNA CONTENT IN SUBMICROSOMAL FRACTIONS PREPARED BY ZONAL CENTRIFUGATION

	Smooth-Surfaced Microsomes	Rough-Surfaced Microsomes
Tube Number	11-21	22-27
Protein*	12.1	11.6
mg/g liver	(11.6, 12.1, 12.5)	(10.5, 11.5, 12.7)
RNA†	3.57	2.87
mg/g liver	(3.50, 3.64)	(2.67, 3.07)
mg/mg protein	0.29	0.24
Phospholipid†	4.65	3.26
mg/g liver	(4.72, 4.58)	(3.22, 3.30)
mg/mg protein	0.38	0.28

* Average of three experiments. Protein ratio of smooth-surfaced microsomal fraction to rough-surfaced microsomal fraction is 1.04.

† Average of two experiments.

proliferation of smooth endoplasmic reticulum in rat liver (5) and the zonal profile plainly reflects this fact.

Our experiments demonstrate that zonal centrifugation subfractionates the microsomal components of rat liver into smooth- and rough-surfaced forms. This system not only offers high capacity and good resolution, but also makes it possible to visualize the distribution of the elements present in the microsomal fraction of a cell as a continuous spectrum of subcellular particles. This technique is being applied in our laboratory to study the zonal profiles of postmitochondrial fractions from Morris hepatomas and other tumors. Preliminary experiments have indicated quantitative differences between neoplastic and normal tissues.

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