ELECTRON MICROSCOPE EXAMINATION OF SUBCELLULAR FRACTIONS

III. Quantitative Analysis of the Microsomal Fraction Isolated from Rat Liver

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ABSTRACT

Rat liver microsomes and microsomal subfractions isolated by density equilibration were submitted to a quantitative morphological and biochemical analysis. The total area of the endoplasmic reticulum was estimated at 7.3 m^2 per g of liver. The microsome fraction contained 2.8 mg of phospholipids and 6.7 mg of proteins per m^2 of membrane area. After correction for ribosomal and intracisternal proteins, the latter value was lowered to 4.7 mg of membrane protein per m^2. More than half of the microsomal vesicles carried ribosomes. After density equilibration of the microsomes, the distribution pattern of ribosomes followed closely that of RNA. The ribosome load of the microsomal vesicles increased steadily along the density gradient, indicating the existence of a continuous spectrum of microsomal entities ranging from entirely ribosome-free vesicles to vesicles heavily coated with ribosomes.

INTRODUCTION

Morphometric studies of the cellular membranes have been carried out on normal rat liver by Loud (29) and by Weibel et al. (47). Such measurements *in situ* offer the advantage of allowing easy cytological identification of the structures, but they do not permit direct correlation between morphological and biochemical data on the same material. Such a correlation can be more directly established when the same subcellular fractions are submitted to the morphometric and the biochemical analysis. We report here the results of a quantitative morphological analysis of rat liver microsomes and of microsomal subfractions isolated by density equilibration in a sucrose gradient. This study parallels a biochemical analysis, the results of which have been briefly reported before (1) and will be described in detail in a subsequent publication[1].

The main purpose of the present work was to investigate the distribution of ribosomes between microsomal vesicles. The analysis of microsome fractions in sucrose gradients has shown that the density distribution of RNA-bearing vesicles is very broad (1, 10, 12, 15). This finding could be interpreted in two ways: (*a*) The ribosome load of rough vesicles is very variable, and this variability accounts for, or, at least, is correlated with, the dispersion of their equilibrium density. (*b*) Rough

[1] Amar-Costesec, A., H. Beaufay, E. Feytmans, D. Thinès-Sempoux, M. Robbi, and J. Berthet. In preparation.

vesicles form a homogeneous population as regards their ribosome load, and the dispersion of their equilibrium density originates from the variability of some other physicochemical characters. In agreement with some evidence presented by others (10, 12, 42), our quantitative morphological data support the former interpretation.

We have also measured the size distribution of the microsomal vesicles, as well as their total membrane area, which were correlated with their chemical composition.

MATERIALS AND METHODS

Subcellular Fractions

Livers from female Wistar rats, weighing about 150 g and fasted for 18 hr, were fractionated according to de Duve et al. (14) with two minor modifications: (*a*) The homogenization and resuspension medium was 0.25 M sucrose buffered at pH 7.4 with 3 mM imidazole-HCl. (*b*) A combined M + L fraction was sedimented from the cytoplasmic extract by an integrated force of 250,000 *g*-min. As described by de Duve et al. (14), the fluffy layer of the M + L pellet was removed with the supernatant fluid. The microsomal (P) fraction was separated by an integrated force of 3,000,000 *g*-min and washed once. It was resuspended carefully by means of a Dounce homogenizer (Kontes Glass Co., Vineland, N. J.) with buffered sucrose, up to a volume of 1 ml per g of tissue.

In one experiment (preparation II), the microsomal fraction was subfractionated by density equilibration in a sucrose gradient. The isopycnic centrifugation was performed in the rotor assembly designed by Beaufay (6), of which the main characteristics and advantages have been summarized by Leighton et al. (27). The rotor running at about 7000 rpm was loaded in the order of increasing density with (*a*) 10 ml of the P fraction, (*b*) 32 ml of a sucrose solution, the concentration of which increased linearly with respect to volume from 23.1% (w/w) sucrose (density 1.10) to 52.2% (w/w) sucrose (density 1.24), and (*c*) 6 ml of a cushion of 67.4% (w/w) sucrose (density 1.34). All solutions were buffered with 3 mM imidazole-HCl pH 7.4. With the centrifugation cell used, the inner and outer radial distances of the ring containing the microsomal sample were 5.30 and 5.55 cm, respectively, and the gradient itself extended from 5.55 to 6.29 cm. Owing to the short distance to be travelled by the microsomal vesicles, a centrifugation time of 3 hr at 35,000 rpm was sufficient to bring them very close to their equilibrium position. At the end of the centrifugation, 15 fractions were delivered automatically from the decelerating rotor, between 9000 and 6000 rpm. The densities of the fractions were measured as described previously (27).

Biochemical Methods

Proteins were determined by the method of Lowry et al. (30), adapted to the Technicon Auto-Analyzer (Technicon Corporation, Ardsley, N. Y.) as described by Leighton et al. (27), with minor modifications. Results are expressed in bovine serum albumin equivalents (Poviet Producten N.V., Amsterdam, Holland).

Phospholipid phosphorus was determined on lipid extracts (7). The weight of phosphorus was multiplied by 25 to give the corresponding weight of phospholipids.

RNA was estimated by the method of Schneider (35), except that the material precipitating in cold 5% trichloroacetic acid was washed three times to effectively remove sucrose, which reacts with orcinol. The standard was yeast RNA (Schwarz Bio Research Inc., Orangeburg, N. Y.). The percentage of RNA in the standard was evaluated from its content in organic phosphate and from its ultraviolet absorption after alkaline hydrolysis (34). The amount of DNA in the RNA standard was negligible (35).

Glucose 6-phosphatase was assayed according to de Duve et al. (14).

Morphological Procedures

Fixation

Particulate suspensions in 0.25 M sucrose were fixed by mixing with 20–50 times their volume of an ice-cold solution of 1.5% glutaraldehyde (Fluka AG, Basel, Switzerland) in 0.05 M phosphate buffer, pH 7.2. In the case of subfractions obtained by density equilibration, osmotic shock to the unfixed particles was avoided by first adding 0.25 volume of 7.5% glutaraldehyde in 0.05 M phosphate buffer; 30 min later, the suspensions were further diluted with 1.5% glutaraldehyde in 0.05 M phosphate.

Filtration

The microsomal particles were collected on Millipore filters of the VF type (Millipore Corporation, Bedford, Mass.) as described by Baudhuin et al., (5), with two modifications: (*a*) The amount of material filtered was decreased to about 40 μg of protein. It was thus possible to photograph the whole thickness of the pellicle at a suitable magnification. Moreover, excessive packing of the particles close to the filter was avoided. (*b*) After filtration, the pellicle was covered with a naked Millipore filter instead of a filter coated with a layer of red blood cells. This procedure proved more convenient, but still efficient, to prevent losses of particles during subsequent handling.

When this work was started, the average pore size of the Millipore VF filter was claimed by the manufacturer to be 10 nm, which is well below the diameter of ribosomes. Subsequently, however, the pore size was reevaluated and was shown to be close to 25 nm. (This type of filter is now designated as the VS filter.) The possibility of losing ribosomes through the filter thus deserved consideration. It was investigated in the following way: Tritiated orotic acid was injected into a rat 4 hr before isolating the microsomal fraction. A pellicle was prepared in the usual manner and the filtrate was collected. No more than 5% of the radioactivity of the sample was recovered in the filtrate. The loss of ribosomal material was thus quite small.

Postfixation, Dehydration, and Embedding

The pellicles were postfixed with osmium tetroxide, dehydrated in alcohol, and embedded in Epon (Ladd Research Industries, Inc., Burlington, Vt.) as previously described (5), except that rinsing in 0.05 M phosphate buffer pH 7.2 after osmium tetroxide treatment was extended overnight. Epon was polymerized for at least 2 days at 60°C.

Sectioning and Measurement of Section Thickness

Grey sections were cut perpendicular to the surface of the pellicle (5), with an LKB Ultrotome I microtome equipped with a diamond knife, and picked up on naked 200-mesh copper grids. The thickness of the sections was measured as described by Gillis and Wibo (17). Sections 40–45 nm thick were located on the grid and selected for electron microscope examination.

Electron Microscope Examination

The sections were stained by immersion in uranyl acetate (saturated aqueous solution, diluted twice with 50% alcohol) for 2 min, then with lead citrate (33) for 5 min. They were coated with a thin carbon layer to improve their stability in the electron beam.

The microscope was a Siemens Elmiskop I, equipped with an anticontamination device. The micrographs were taken at a primary magnification of 14,000–20,000. The magnification was determined by means of a grating replica (54,800 lines per inch, E. F. Fullam, Inc., Schenectady, N. Y.), as described by Baudhuin and Berthet (4). Positive prints were made on Copyline film or Brovira paper (Agfa-Gevaert, Leverkusen, Germany). The final magnification was 60,000. The dimensional stability of the photographic paper was checked and the distortion was found to be smaller than 2%.

Stereologic Analysis

Size Distribution of Vesicles

The microsomal profiles were classified according to size by means of a Zeiss TGZ 3 particle dimension analyzer. The micrographs were scanned over the whole thickness of the pellicle, from its upper surface to the Millipore filter. All closed profiles were included in the dimension analysis, except those that were obviously mitochondrial, lysosomal, or peroxisomal. Open profiles were disregarded. In some instances, rough and smooth profiles were recorded separately.

The dimension analyzer is constructed for the measurement of circular profiles. When the microsomes are suspended in 0.25 M sucrose, a good number of profiles are indeed close to circular (Fig. 2). However some are elongated or show "tails" or invaginations of their limiting membrane. The registered radius is then that of the circle which has the same area as the distorted profile. Despite this approximation, the method seems to be applicable for microsomes in 0.25 M sucrose, as will be shown in the next section. On the other hand, similar measurements could not be made on density gradient subfractions, because of the marked distortion suffered by the microsomal vesicles when exposed to hypertonic sucrose (Fig. 6).

Like all isolated subcellular particles embedded in plastic media, microsomal membranes appear as opaque bodies against a transparent background. The radii of the nonequatorial profiles are thus overestimated (21, 46). The error is appreciable in this case, since the mean diameter of the vesicles is only about three times the section thickness. Another bias arises from the impossibility of identifying the smallest profiles, especially poorly contrasted polar sections. The methods developed by Baudhuin and Berthet (4) and Baudhuin (3) allow one to correct the measurements for these systematic errors. Automated mathematical procedures were used to compute from the observed size distributions of the profiles the corresponding size distributions of the vesicles, as well as the corrected histograms of the profiles that would have been observed if the sections were infinitely thin and if all the profiles were identified.

Once the distribution of vesicle radii had been computed, the distributions of their surface areas and of their volumes, and the corresponding means and standard deviations were easily calculated.

Measurement of Total Membrane Area

For general applicability, the grid method was used (46). A grid of equidistant test lines, with a spacing approximately equal to the mean diameter of the profiles, was superimposed on the micrographs,

and the number of intersections of the grid lines with the profile contours was counted. The result of this count, expressed as I_L, the number of intersections per unit length of test lines, served to calculate S_v, the total membrane area per unit volume of pellicle, by the well known formula (37, 44):

$$S_v = 2 I_L .$$

This method has the advantage of being very rapid and, therefore, of allowing the scanning of relatively large areas of pellicle, thereby averaging out local fluctuations in particle packing and pellicle thickness. However, the value of S_v obtained in this manner is rather grossly overestimated, for the same reason, due to the finite thickness of the sections, that causes the profile radii to be overestimated.

The required correction factor could be estimated directly in the experiments in which size distributions were determined, by application of a second method for the measurement of S_v, based on the measurement of contour lengths. It is known that S_v is directly proportional to the total length of profile contours per unit area of section (37). This measurement was made on both the observed and the corrected size distributions of the profiles, and the ratio of the two values gave K_s, the overestimation factor, which was used to correct the S_v value obtained by the grid method. In such cases where the actual size distribution of the sample was not determined, K_s was taken to be 1.35, the average value found on three microsomal preparations.

We did not use the S_v values derived from the contour lengths (except for estimating K_s), because they applied to smaller areas of section than did those obtained by the grid method, and were therefore more likely to be affected by local conditions of particle packing. The values obtained by the grid method were more representative of the whole pellicle and more reliable statistically. Moreover, they should not be affected by the noncircular shape of some profiles. In this respect, it is perhaps puzzling that, when applied to the same field of the pellicle (prepared in 0.25 M sucrose), the two methods gave quasi-identical results, since the values derived from the profile sizes, implying circular approximation, should be somewhat smaller than those obtained by the grid method. The agreement observed could suggest that we overestimated slightly the area of noncircular profiles in the measurements with the particle size analyzer. Nevertheless it can be concluded that the shape of most profiles was indeed sufficiently close to circular to allow reliable measurement by that method.

As mentioned earlier, some microsomal vesicles contained invaginations. On equatorial sections, they were clearly recognizable and they were included in the estimation of membrane area by the grid method, where they represented about 4% of the total intersection counts. Since they could not be detected on polar sections, it is likely that the actual S_v values are slightly higher than those reported.

The measured S_v values were further converted to total membrane area per milligram of microsomal protein, by means of the known values of pellicle volume (calculated from the measured diameter and thickness) and protein content (amount of microsomal proteins put on the filter and processed into the pellicle).

Calculation of Total Number and Volume of Vesicles

This information could be calculated only in those experiments in which dimension analysis had been performed. The number of vesicles per milligram of microsomal protein was obtained by dividing the corrected membrane area per milligram of microsomal protein by the mean surface area of the vesicles. In turn, the number of vesicles per milligram of microsomal protein, multiplied by the mean vesicle volume, gave the total vesicle volume per milligram of microsomal protein.

Ribosome Counts

The identification of ribosomes raised some problems. Most ribosomes were easily recognized by their size (15–20 nm), their high contrast, and their position along the edge of the rough profiles. But some were superimposed on polar sections of membranes and were less visible. Sometimes, also, the ribosomal images were not as contrasted as normally, probably because only a part of the particle was included in the section (see Fig. 3). The validity of the ribosome counts will be discussed later.

The counts were made on sections of known thickness, and could thereby be expressed in numbers of ribosomes per unit volume, and eventually converted to numbers of ribosomes per milligram of microsomal protein.

RESULTS

Biochemical Data

In Table I are listed some biochemical properties of the three microsomal fractions analyzed. They contained about 75% of the glucose 6-phosphatase activity of the liver, associated with 19% of the proteins, 47% of the phospholipids, and 57% of the RNA. Preparation II was further fractionated by density equilibration. As shown in Fig. 1, the distribution patterns of microsomal proteins and glucose 6-phosphatase are closely similar. They are characterized by a rather low

TABLE I

Biochemical Properties of the Microsomal Preparations

	Preparation			
	I	II	III	Mean
Proteins				
mg in microsomes obtained from 1 g of liver	36.9	44.5	45.8	42.4
% of the liver content*	18.5	21.5	17.3	19.1
Phospholipids				
mg in microsomes obtained from 1 g of liver	16.9	17.6	19.4	18.0
% of the liver content*	46.8	49.1	45.0	47.0
RNA				
mg in microsomes obtained from 1 g of liver	3.70	4.76	3.74	4.07
% of the liver content*	56.2	66.2	48.7	57.0
Glucose 6-phosphatase				
Units (μmoles per min) in microsomes obtained from 1 g of liver	16.3	15.6	21.5	17.8
% of the liver content*	71.8	78.3	76.1	75.4

* Liver content is defined here as the sum of the amounts recovered in the fractions (N, M + L, P, and S [14]). The recoveries were about 100% for proteins, 90–95% for glucose 6-phosphatase, and 85–105% for phospholipids and RNA.

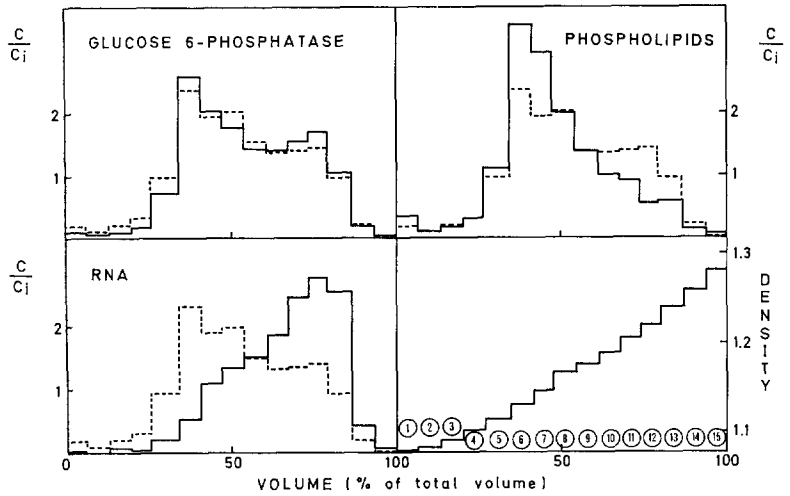

FIGURE 1 Isopycnic distributions of some microsomal constituents (Preparation II) in sucrose-H_2O gradient. C/C_i is the relative concentration, i.e. the ratio of the concentration C in the fraction to the concentration C_i that the constituent would have if it were uniformly distributed in the whole gradient. The distribution pattern of proteins is superimposed (broken line) on those of glucose 6-phosphatase, phospholipids and RNA (solid line). The density of the subfractions is given in a separate graph together with their number. With respect to the microsomal sample layered on the gradient, the recoveries were: glucose 6-phosphatase, 104%; phospholipids, 109%; RNA, 77%; proteins, 108%.

modal equilibrium density (1.13) and by a marked shoulder in the dense RNA-rich region, which in the case of the enzyme, forms a second peak. The distribution pattern of phospholipids exhibits the same modal density, but is less asymmetrical. Although RNA is mainly concentrated in the dense subfractions, its distribution pattern overlaps considerably those of the other components.

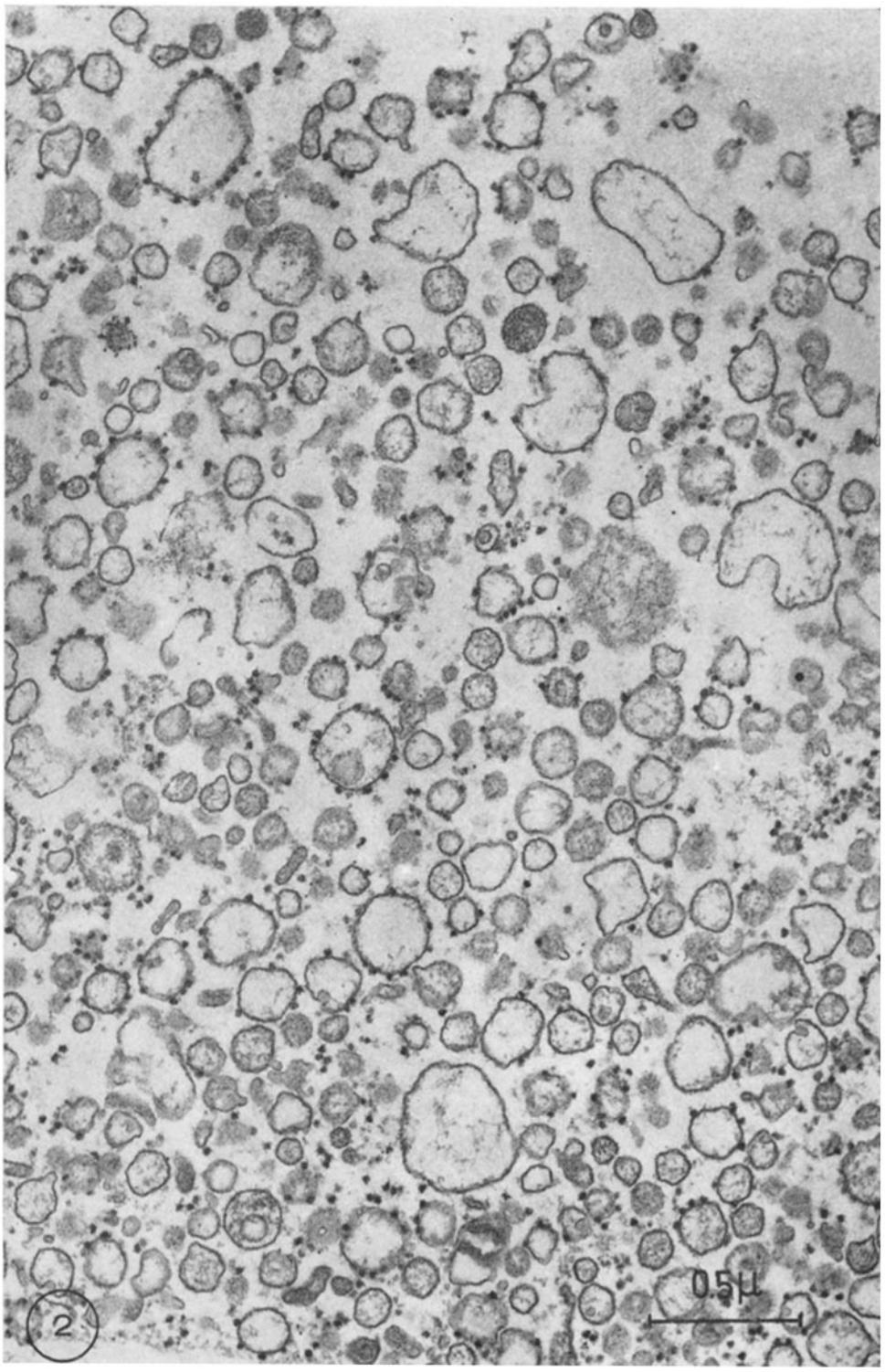

FIGURE 2 Appearance of a microsomal pellicle. The boundary between the pellicle and the Millipore filter is visible in the lower left-hand corner. × 42,500.

These results are in agreement with the more extensive biochemical study made by Amar-Costesec et al. (1).

Morphological Analysis of the Microsomal Fraction

The appearance of a microsomal pellicle is illustrated in Fig. 2. It is made up largely of closed, approximately circular, vesicular structures of both the rough- and the smooth-surfaced variety. Some vesicles show a content of moderate electron opacity. Most ribosomes seem to be attached to membranes. Two areas of this micrograph are enenlarged in Fig. 3, to demonstrate how the various profiles were classified in our quantitative analysis.

Fig. 4 shows the size distributions of the microsomal profiles and vesicles in the three preparations. As can be seen in the upper half of the figure, the corrected profile histograms differ markedly from the uncorrected ones. Obviously, there is a considerable overestimation of the profile sizes due to the finite section thickness. On the other hand, few small profiles seem to have been neglected in the systematic scanning of the pellicle.

In Table II are listed the main characteristics that could be derived from the size distributions. The K_s factors, estimated as explained in Materials and Methods, are also given. The variability of the microsomal preparations appears rather small.

In the case of preparation III, rough and smooth profiles were registered separately and the corresponding histograms were constructed (Fig. 5 and Table III). The smooth profiles tend to be somewhat smaller on an average than the rough ones. The two histograms are significantly different ($P < 0.01$) when compared by the Kolmogorov-Smirnov test (24). Preliminary observations on serial sections indicate that some 20% of the smooth profiles originate from rough vesicles. When the profile distributions in a given section are corrected by taking into account the true nature of the vesicles as revealed by the adjoining sections, the difference in average size between rough and smooth vesicles persists.

In Table IV are shown the total areas per milligram of microsomal protein for the various types of microsomal membranes, measured by the grid method. Each pellicle was scanned over a distance of 15 μm along its surface, a distance sufficient to ensure good statistical sampling. It is seen that the total area of rough vesicles is slightly lower than that of smooth vesicles. However, it must be remembered that the latter are overestimated by about 20% at the expense of the former. Open membrane fragments represent less than 2% of the total area of microsomal membranes. Their exclusion from the dimension analysis cannot have affected the computed distributions significantly.

Also shown in Table IV are the numbers and total volumes of microsomal vesicles per milligram

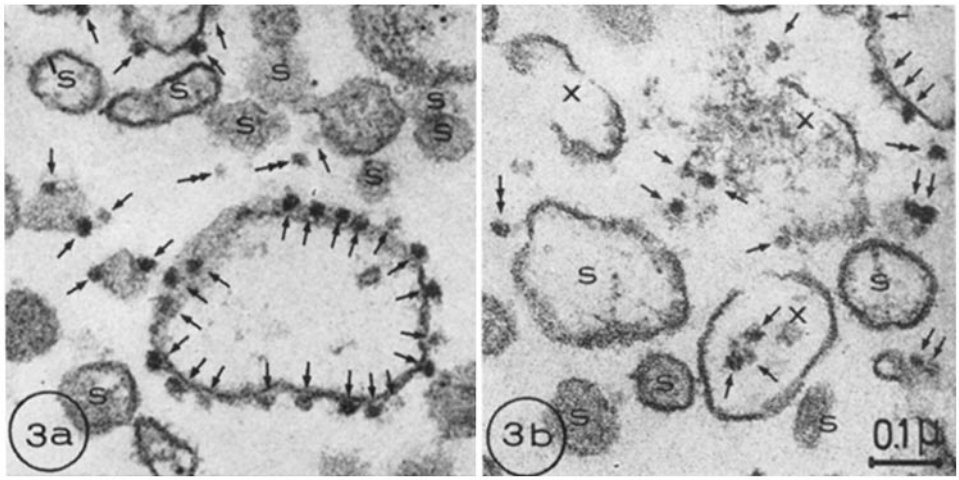

FIGURE 3 Enlargements of two areas of the micrograph presented in Fig. 2. Single arrows point to ribosomes attached to membranes. Double arrows point to ribosomes that are apparently free. All objects that were counted as ribosomes in these areas are indicated. *S*, smooth profiles. *X*, open profiles. × 90,000.

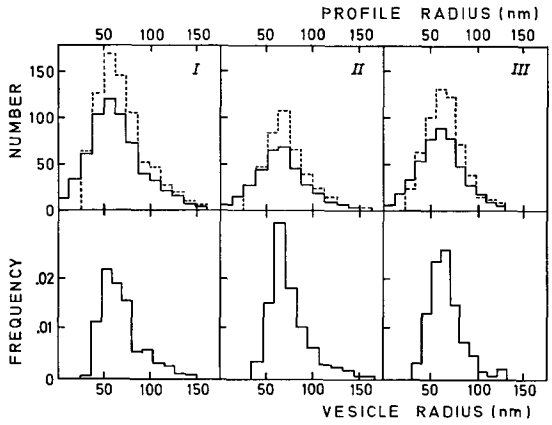

FIGURE 4 Size distributions of microsomal profiles and vesicles in the three experiments. In the upper diagrams, the broken line gives the histogram constructed from the experimental data, whereas the continuous line represents the corrected size distribution of the profiles. The lower diagrams give the frequency distributions of the vesicle radii. The dimension of the frequency scale is nm^{-1}.

TABLE II

Dimension Analysis of the Microsomal Profiles and Vesicles

	Preparation			
	I	II	III	Mean
Number of profiles measured	767	436	626	
Mean radius of the vesicles, nm	69.1	70.3	63.6	67.7
Standard deviation of radius, nm	24.0	21.8	19.6	21.8
Mean area of the vesicles, $nm^2 \times 10^{-4}$	6.72	6.8	5.57	6.36
Mean volume of the vesicles, $nm^3 \times 10^{-6}$	1.97	1.95	1.43	1.78
Sum of the contour lengths of the profiles, μm				
uncorrected	314.3	177.7	233.3	
corrected	236.7	132.2	167.6	
Area overestimation factor, K_s	1.33	1.34	1.39	1.35

of microsomal protein that could be computed from the size distributions and area measurements. Finally this table includes the numbers of ribosomes that were counted in the microsomal preparations. They are expressed in terms both of total microsomal protein and of rough membrane area. Implicit in the latter calculation is the assumption that all microsomal ribosomes can be considered as membrane-bound. That this must be true for at least 95% of the ribosomes present is supported by the following arguments: (*a*) Careful examination of the electron micrographs shows that the membrane-bound character is questionable for about 15% of the microsomal ribosomes. Since a number of them are no doubt attached to membranes that are not included in the same section, the percentage of free ribosomes in the microsomal preparations should be smaller than 15%. (*b*) The centrifugation conditions used for separating the microsomes from the postmitochondrial supernatant, do not suffice to sediment an important fraction of the free ribosomes which, according to Blobel and Potter (8), amount to no more than 25% of the ribosomes in rat liver homogenates. (*c*) Although free ribosomes have a very high density (1.41 [18]), only traces of RNA are detected at a density higher than 1.26 after equilibration of our microsome preparations in a sucrose gradient, and the distribution pattern of RNA does not change appreciably with respect to that of other microsomal constituents after more prolonged centrifugation (1).

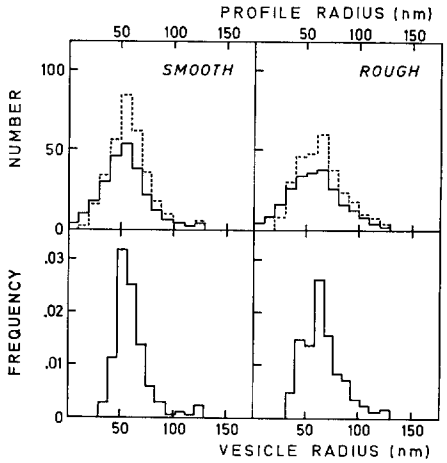

FIGURE 5 Size distributions of smooth and rough elements of Preparation III. Same representation as in Fig. 4.

TABLE III

Dimension Analysis of the Smooth and Rough Elements of Preparation III

	Smooth	Rough
Number of profiles measured	331	295
Mean radius of the vesicles, nm	61.8	65.9
Standard deviation of radius, nm	18.5	20.8
Mean area of the vesicles, $nm^2 \times 10^{-4}$	5.23	5.99
Mean volume of the vesicles, $nm^3 \times 10^{-6}$	1.31	1.59
Sum of the contour lengths of the profiles, μm		
uncorrected	118.4	114.9
corrected	84.6	83.7
Area overestimation factor, K_s	1.40	1.37

Morphological Analysis of the Microsomal Subfractions Obtained by Density Equilibration

MORPHOLOGICAL APPEARANCE

Fig. 6 presents typical micrographs of subfractions *3–14* from the experiment shown in Fig. 1. Particularly striking is the increase in ribosome frequency with the density of the subfractions. None of the subfractions seems to contain a sizable proportion of free ribosomes. As the sucrose medium becomes more hypertonic, the rough profiles increasingly deviate from the circular shape.

The three lightest subfractions are composed of very heterogeneous structures, some of which are reminiscent of Golgi elements and vacuoles containing lipoprotein granules. Open membrane fragments are fairly frequent. Subfractions *8* and *9* contain numerous opaque, often elongated, structures, the significance of which remains unknown, and a few mitochondrial profiles. In subfractions *7–12*, relatively large granules, devoid of ribosomes and showing a filamentous content, are visible. Most of them could be peroxisomes partially depleted of their matrix material. Typical core-containing peroxisomes and free cores are present in the densest fractions. Very few lysosomes can be recognized in any of the fractions.

CORRELATION BETWEEN BIOCHEMICAL AND MORPHOMETRIC DATA

The nine main subfractions (*6–14*) were submitted to quantitative morphological analysis. Taken together, they represent 98% of the RNA, 87% of the phospholipids, and 90% of the proteins recovered in the gradient.

Each pellicle was scanned along its surface over a distance of about 6 μm. The membrane areas were estimated by the grid method and the ribosomes were counted. The results are represented graphically in Fig. 7, together with the distributions of phospholipids, RNA and nonribosomal proteins. Altogether, the membrane areas and the ribosomes recovered in the nine subfractions amounted to 94 and 96%, respectively, of the quantities layered on top of the gradient.

Our analysis discloses a good agreement between the morphological and the biochemical characters of the subfractions. The distribution pattern of the membrane area is intermediate between those of proteins and phospholipids; the ribosome number is proportional to the RNA content.

DISTRIBUTION OF RIBOSOMES ON THE MICROSOMAL MEMBRANES

For each subfraction analyzed, at least 400 profiles were classified according to the number of ribosomes bound. All profiles in a given section area were included, irrespective of their size and whether apparently polar, equatorial, or intermediate in origin. The results are presented in Fig. 8. The width of the histograms obviously reflects both the dispersion of the sizes of the profiles and that of the numbers of ribosomes per unit area of

TABLE IV

Total Area, Number and Total Volume of Vesicles, and Number of Ribosomes, in the Microsomal Fractions

	Preparation				
	I	II	III	Mean	Corrected mean*
Number of intersections counted	2,852	2,882	2,670		
Number of ribosomes counted	2,951	3,489	3,166		
Total area of smooth vesicles, m^2/mg protein	0.134	0.093	0.099	0.109	0.081
Total area of rough vesicles, m^2/mg protein	0.105	0.080	0.098	0.094	0.070
Total area of all vesicles, m^2/mg protein	0.239	0.173	0.197	0.203	0.151
Total area of open membrane fragments, m^2/mg protein	0.004	0.004	0.002	0.003	0.003‡
Number of vesicles per 10^{-12} mg protein					2.37
Total volume of vesicles, μl/mg protein					4.22
Number of ribosomes per 10^{-13} mg protein	1.78	1.61	1.61	1.67	
Number of ribosomes per μm^2 of rough membrane	170	201	164	178	240

* The uncorrected mean areas were divided by 1.35 (K_s).

‡ Since most open profiles arise from membrane fragments sectioned normally (the open character of the profile is not recognizable on polar sections), no correction for overestimation was applied.

membranes. Nevertheless the pattern remains unimodal and, as the density of the subfraction becomes higher, it shifts regularly to higher numbers of ribosomes per profile. This relationship is also evident from Fig. 9, in which the mean ribosome numbers per profile are plotted against the densities of the subfractions.

Again, this result could mean that the average size of the profiles increases along the density gradient as well as the number of ribosomes per unit area of the membranes. We found that differences in average size of the profiles cannot account for the relationship of Fig. 9. By means of the grid method, the mean perimeter of rough profiles was determined in subfractions *7*, *10*, and *13*. It was only 1.24 and 1.29 times larger in fractions *10* and *13* than in fraction *7*, whereas the mean ribosome number per profile was, respectively, 1.87 and 3.79 times higher.

The average number of ribosomes attached to 1 μm^2 of rough membrane is given in Fig. 10 for each subfraction. If the smooth profiles derived from rough vesicles could be taken into account, the curve would be somewhat lower than that shown in Fig. 10, especially for the light subfractions where the number of ribosomes per rough vesicle is small. Correction for this cause of error would only increase the slope of the experimental curve, which confirms the fact that the number of ribosomes per rough vesicle increases with the density of the subfraction. Were it not so, the average number of ribosomes per 1 μm^2 of rough membrane would remain constant and only the proportion of rough vesicles would increase with the density of the subfraction.

DISCUSSION

Surface Area of the Endoplasmic Reticulum Membranes

On the average, the microsomes isolated from 1 g of liver contain 42.4 mg of protein (Table I) and have a membrane area of 0.154 m^2 per mg of protein (Table IV). The total membrane area of the fraction is thus 6.5 m^2 per g of liver.

Rough and smooth elements of the endoplasmic reticulum (ER) are the major, but not the only, component of the microsomal fraction from rat liver. Correction for the other components must first be made before the area of the ER membranes

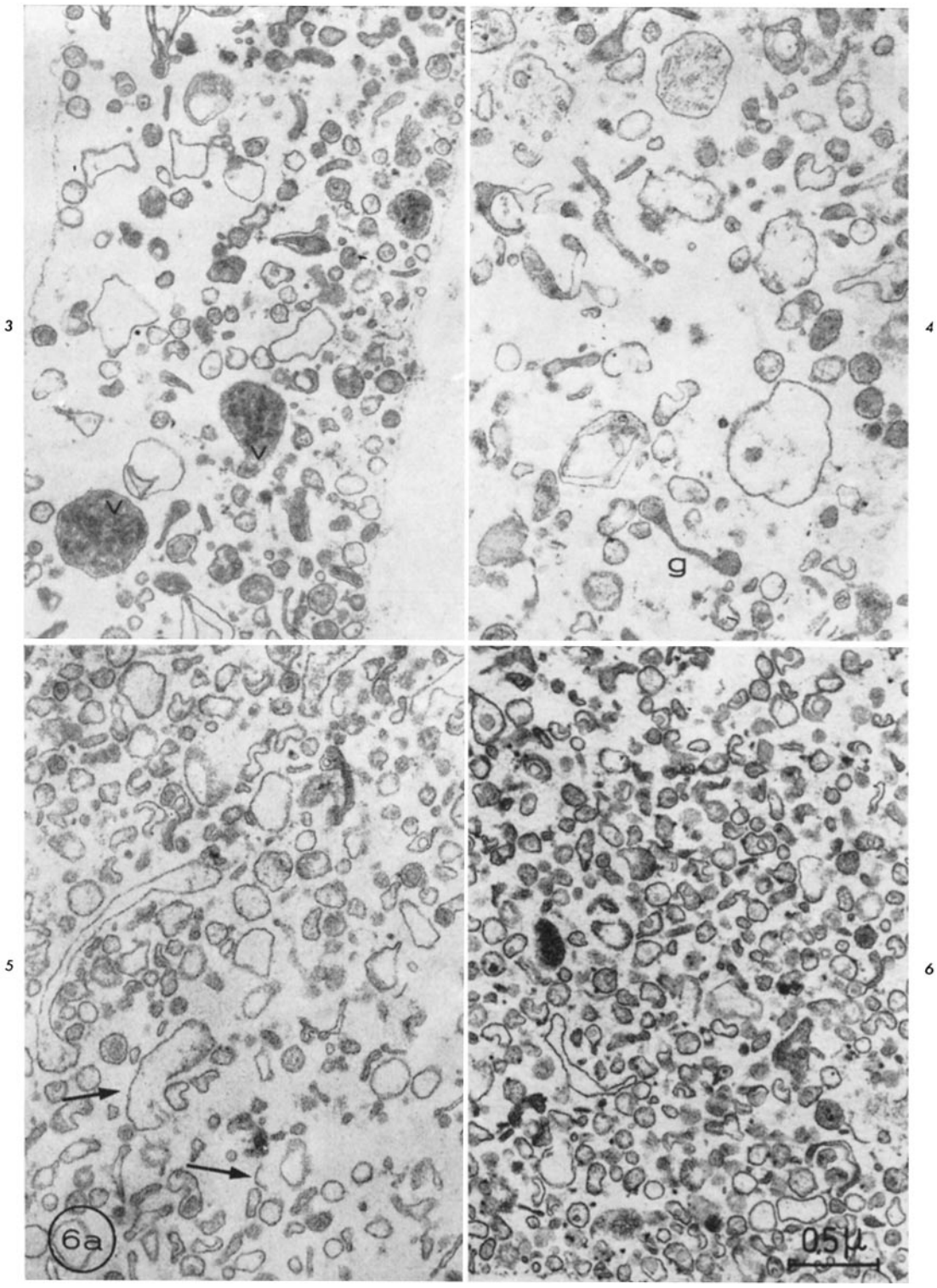

FIGURE 6 Morphology of the microsomal subfractions obtained by density equilibration. They are presented in the order of increasing density. The number beside each picture gives the position of the subfraction in the gradient (see Fig. 1). × 30,000.

FIGURE 6a Subfractions *3–6*. Note vacuoles containing lipoprotein granules (*v*) in subfraction *3*, at least one Golgi element (*g*) in subfraction *4*, open membrane fragments in subfraction *5* (arrows).

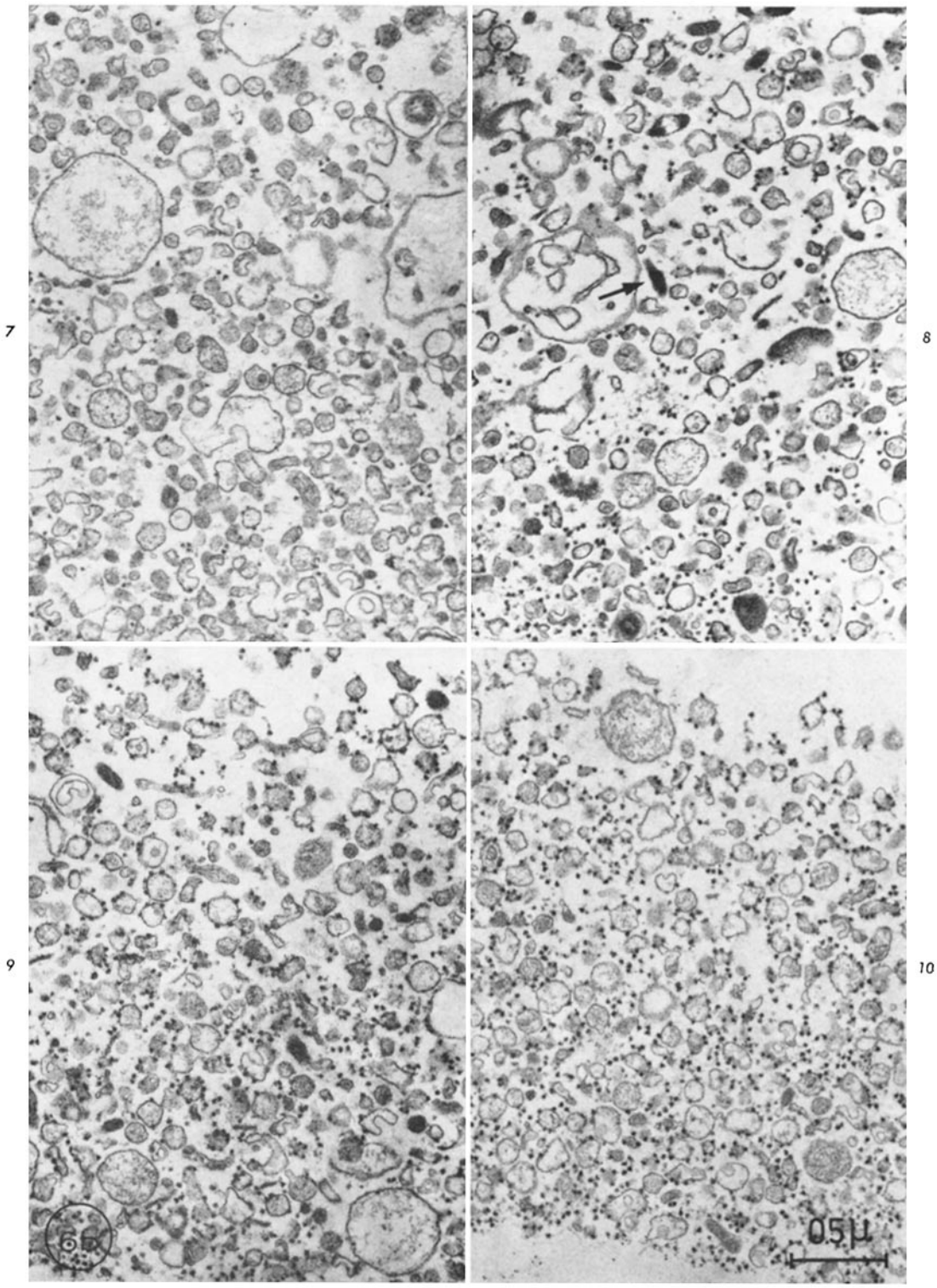

FIGURE 6b Subfractions *7–10*. Note the progressive increase of the number of ribosomes. Several large granules, containing a filamentous material, are visible, as well as many opaque structures (mainly in subfraction *8*; for example, arrow).

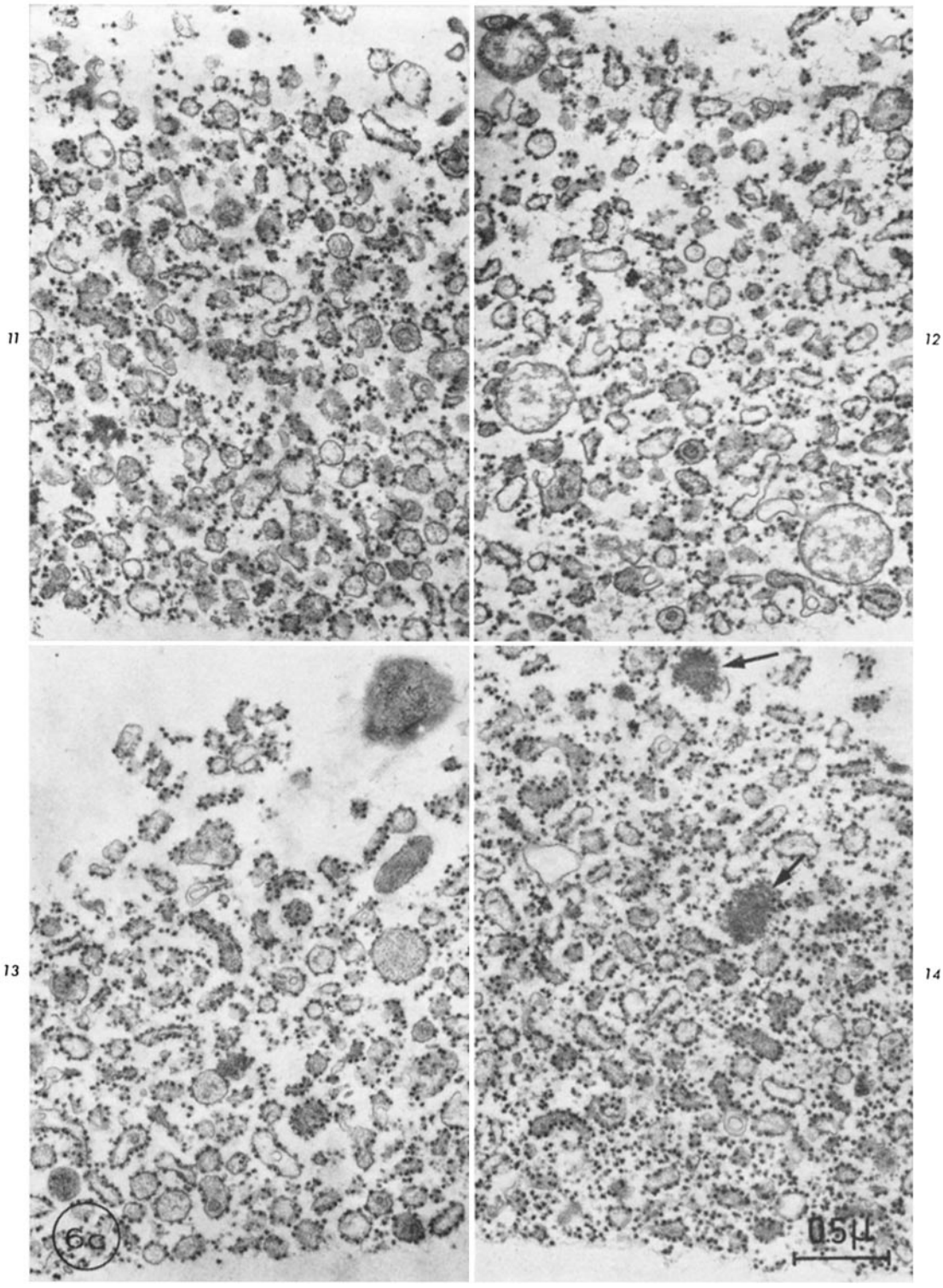

FIGURE 6c — Subfractions *11–14*. Note the progressive flattening of the rough profiles. The ribosome load becomes very important. Two free peroxisomal cores can be seen in subfraction *14* (arrows).

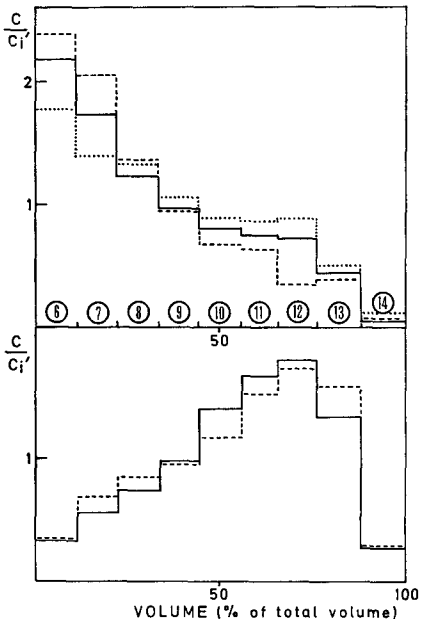

FIGURE 7 Correlation between the morphometric and the biochemical characters of the microsomal subfractions. The distribution patterns of membrane areas (——), phospholipids (- - - -), and nonribosomal proteins (. . . .) are shown in the upper diagram; those of ribosomes (——) and RNA (- - - -) in the lower one. The biochemical data are from Fig. 1. The nonribosomal proteins were estimated by subtracting from the total proteins of the fractions an amount equal to 80% of their RNA content. All data are normalized, C being the amount per volume of subfraction, C'_i the corresponding value in the pooled subfractions.

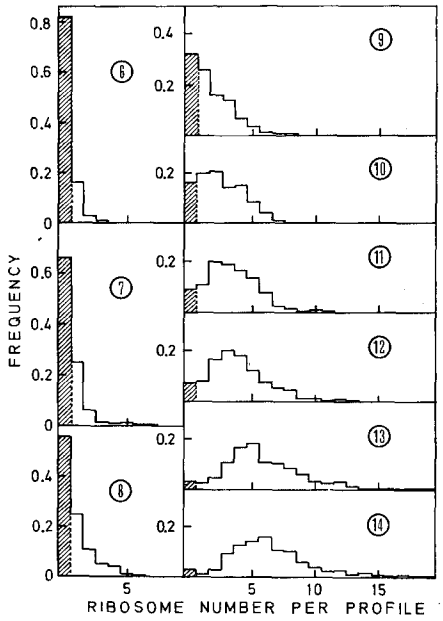

FIGURE 8 Frequency distributions of the number of ribosomes per profile. The shaded areas correspond to smooth profiles.

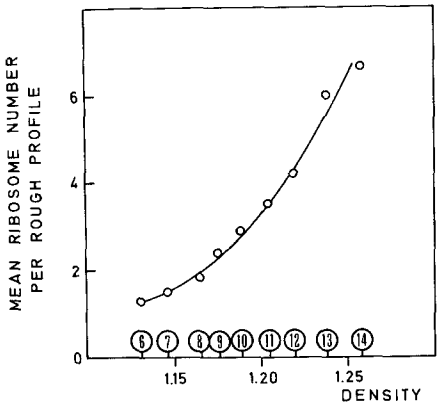

FIGURE 9 Mean ribosome number per rough profile in relation to the density of the subfraction.

can be deduced from the area of microsomal membranes.

The microsomal fraction includes a special membrane component closely related to the plasma membrane (43, 48). It has been estimated that this component represents about 10% of the microsomal membranes (0.65 m^2/g of liver).[2] The microsomal monoamine oxidase is carried by yet another component (1, 43). If the monoamine oxidase-bearing structures are related in some way to the outer mitochondrial membrane, their area of membrane may be evaluated at 0.2 m^2/g of liver, since (*a*) 20% of the total monoamine oxidase is recovered in the microsomal fraction (1), and (*b*) the outer mitochondrial envelope has an area of about 1 m^2/g of liver (3, 4). The smooth elements derived from the Golgi complex are another minor component of the microsomes.

By subtracting the areas attributed to these minor microsomal components, we find the membrane area of the ER recovered in the P fraction to be no greater than 5.65 m^2/g of liver. If this result is extrapolated to the whole homogenate, by taking the percentage of glucose 6-phosphatase

[2] Amar-Costesec, A., D. Thinès-Sempoux, M. Wibo, H. Beaufay, M. Robbi, and J. Berthet. In preparation.

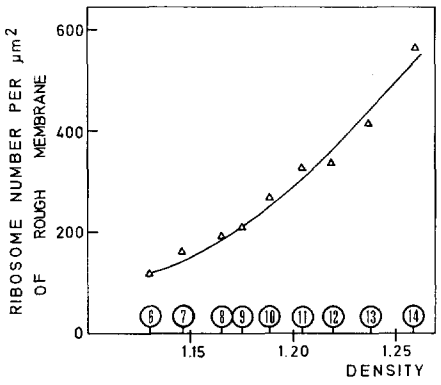

FIGURE 10 Average ribosome number per μm^2 of rough membrane in relation to the density of the subfraction. The experimental areas have been divided by 1.35, the overestimation factor determined on the total microsomal preparation in isotonic sucrose.

recovered in the microsomal fraction as equal to that of the ER membranes, the total area of the ER membranes would be of the order of 7.5 m^2/g of liver (7.9 m^2/ml).

This value is to be compared with those obtained by Loud (29) and by Weibel et al. (47) on intact liver. The figure reported by Loud (29) is 5.5 m^2 of ER membrane per ml of cytoplasm of parenchymal cell, or 4.3 m^2/ml of liver, since the hepatocyte cytoplasm occupies 78% of the whole liver volume (47). This value refers to the membranes that were actually detected on the micrographs, and must be corrected for the membranes that were sectioned at too small an angle with respect to their plane to be recognizable. According to Loud (29), the correction factor is of the order of 1.5. The corrected estimate thus becomes 6.5 m^2/ml of liver, a value not too far removed from ours.

The value given by Weibel et al. (47) is considerably larger, 10.9 m^2/ml of liver, which, according to the authors, must be further multiplied by 1.25 to account for unrecognized profiles, giving 13.6 m^2/ml of liver. It is known that the development of the endoplasmic reticulum can suffer great variations according to strain, diet, and other environmental factors. However, it seems unlikely that differences of this sort explain the large discrepancy between our estimates of membrane area and those of Weibel et al. (47), since the chemical data of the Swiss authors seem to agree with ours. This point will be discussed further in the next section.

Phospholipid and Protein Content of Microsomal Membranes per Unit Area

Since we have no information on the chemical composition of the different types of membranes present in the microsome fraction, we can only relate the microsomal phospholipids and proteins to the area of the whole of the microsomal membranes. In first approximation, these chemical characters apply to the ER membranes, which represent the major membranous component of microsomal preparations. In line with this interpretation, cholesterol has been disregarded in this study, since the endoplasmic reticulum of the liver contains little, if any, cholesterol (43, 48).

If it is assumed that microsomal phospholipids are essentially structural constituents of membranes, the average phospholipid content of the microsomal membranes is 2.8 mg/m^2. If the phospholipids exist as molecular bilayers in the membranes, an average phospholipid molecule occupies an area of 0.95 nm^2, a value which is fairly close to the most reliable estimates obtained on natural and artificial membranes (39). In the case of the phospholipids of the plasma membrane of the erythrocyte, a cross-sectional area of 0.875 nm^2 was indeed calculated on the assumption that they form a bimolecular leaflet (2). Similarly, a value of about 1 nm^2 can be deduced from the recent data of Kleinig (26) on nuclear membranes.

The protein content of the microsomal membranes can be evaluated by subtracting the ribosomal and luminal proteins from the total microsomal proteins (42.4 mg/g of liver). We may assume that the amount of ribosomal proteins is equal to 80% of that of RNA (19, 40) i.e. 3.3 mg/g of liver. Although the amount of luminal proteins was not measured, an estimate of about 20% of the microsomal proteins is usually considered reasonable (11). On an average, our microsomal preparations would thus contain about 30.6 mg of membranous proteins per g of liver, or 4.7 mg/m^2.[3] The specific content in phospholipids being 2.8 mg/m^2, the bulk of the membrane constituents would amount to 7.5 mg/m^2. Assuming that the density of the

[3] It must be emphasized that these protein values were calculated from the amounts of serum albumin that give the same coloration in the Lowry method as the microsomal sample assayed. The actual amounts could be appreciably different, if the amino acid composition of microsomal proteins differed markedly from that of serum albumin.

structure is 1.15, the volume occupied by the proteins and phospholipids would be 6.5 $\mu g/m^2$ and the thickness of an average microsomal membrane 6.5 nm. This value is in fair agreement with electron microscope determinations of the thickness of the ER membranes, which range from 5 to 8 nm (20, 23, 28, 31, 36, 50).

These calculations suggest that our morphometric data are not seriously in error, leaving as sole questionable procedure their extrapolation to the whole homogenate on the basis of the percentage of the glucose 6-phosphatase activity recovered in the microsome fraction. This extrapolation is admissible since all enzymes specifically associated with the ER membranes are present in the P fraction to approximately the same extent (1).

As was pointed out above, Weibel et al. (47) have reported an estimate of the membrane area of the endoplasmic reticulum that is some 1.7 times greater than ours. Apparently, therefore, their animals must have a more highly developed endoplasmic reticulum than have ours. This would be an acceptable explanation, but for the chemical results reported by Staübli et al. (38). The authors prepared microsomal fractions from the livers that were submitted to morphometric analysis. They give the phospholipid content of their microsomes as 43.4 mg/100 g body weight, the relative liver weight as 3.6 g/100 g body weight, and they assume further in their calculations that they recover about 50% of the "total microsomes" in their fractions. We derive from their data a value of 24.1 mg/g of liver, for the phospholipids associated with the total microsomes. This is almost identical to our own estimate, which, after correction on the basis of glucose 6-phosphatase (Table I), amounts to $18/0.754 = 23.9$ mg/g of liver. Obviously, something must be wrong somewhere, since it is difficult to admit that the phospholipid content per unit area of ER membranes may vary by a factor of 1.7. One explanation for the discrepancy could be that the Swiss workers isolate only 30% of the total ER material in their microsomal fraction. Unfortunately, they give no enzyme data that would have allowed an estimation of this yield. On the whole, one cannot help suspecting that some systematic difference distinguishes the manner in which membrane areas are estimated in Weibel's laboratory and in our own, since the values given by Weibel et al. (47) for the mitochondrial membranes *in situ* are also considerably larger than those reported by Baudhuin and Berthet in mitochondrial preparations (4).

The partial dissociation between proteins and phospholipids in density equilibration experiments (Fig. 1) raises the question of the chemical heterogeneity of ER membranes. It is obvious from Fig. 7 that the dissociation is not simply a reflection of the presence of ribosomal proteins on the rough vesicles. But, it could be that the non ER membranes present in the low density fractions (1, 43) have a higher phospholipid-to-protein ratio than the ER membranes themselves. It is also possible that the denser vesicles contain more luminal proteins than do the less dense ones. Until information on these points becomes available, it would be unwise to conclude that the chemical composition of ER membranes changes with their ribosome load.

Ribosome Counts

As illustrated in Fig. 3, recognition of ribosomal profiles is often difficult and the criteria that have been used in this kind of work are somewhat arbitrary. Comparison with other data nevertheless suggests that our estimates are in the correct range.

In our microsomal preparations, the average surface density of ribosomes on rough membranes was 240 per μm^2. The value obtained by Weibel et al. (47) on intact liver was 334. The significance of this discrepancy is difficult to assess, since the counting procedure used by Weibel et al. was not directly comparable to ours. In particular, the thickness of the sections was not measured in their studies.

One advantage of working on isolated fractions is that the number of ribosomes counted can be compared directly to the amount of RNA determined chemically. In our preparations, we found, per milligram of microsomal protein, 1.67×10^{13} ribosomes (Table IV), and 0.096 mg of RNA (Table I). From these two values, we calculate an apparent RNA content of 5.74×10^{-15} mg of RNA per ribosome. This value is distinctly higher than that of 4.68×10^{-15} given by Tashiro and Yphantis (41) and Tashiro and Siekevitz (40) for guinea pig liver ribosomes; it differs even more from the value of 3.87×10^{-15} that can be deduced from the data of Hamilton and Ruth (19) on rat liver ribosomal subunits. Consequently, our ribosome counts account for only 82% or 67% of the RNA measured, depending on which value

is adopted for the RNA associated with one ribosome.

Part of the remaining 20–30% can be attributed to transfer RNA and messenger RNA linked to the microsomal ribosomes. As shown by Wilson and Quincey (49), these species could amount to 5–10% of the microsomal RNA. Another kind of RNA is to be considered in microsomal preparations, the so-called "membrane RNA". Its existence, which has been supported recently by several authors (9, 16, 22, 25, 45), appears less likely since it was shown that the RNA of apparently smooth membrane preparations can be recovered nearly completely as typical ribosomal RNA (25, 32) and that ribosomal RNA can be easily degraded in various conditions, giving rise to artificial species of RNA (32, 49).

As mentioned earlier, less than 5% of microsomal RNA passed through the Millipore filter during the filtration process. A few free ribosomes or ribosomal subunits could thus have been lost at this stage. Therefore, we are left with 5–20% of unaccounted microsomal RNA, which probably belong to ribosomes that we were unable to discern on the micrographs. These could be superimposed ribosomes, or ribosomes that were incompletely included in the section. The latter were certainly quite frequent in our sections, the thickness of which was about two ribosomal diameters. However we do not know what proportion of a ribosome had to be contained in the section for it to be recognized. Depending on whether this proportion were smaller than, equal to, or greater than, 50%, the numbers of ribosomes per unit volume would be overestimated, correct, or underestimated. Superimposed ribosomes, on the other hand, could occur within polysomes, where the center-to-center distance between ribosomes is about 30–35 nm. Superimposition could thus account for some unnoticed ribosomes, since most liver ribosomes are grouped in polysomes.

In thicker sections, superimpositions are expected to be more important, whereas the proportion of ribosomes that are only partly included in the section decreases. Identical numbers of ribosomes per unit volume were calculated from counts made on two adjacent sections of different thickness (42 and 61 nm). The most likely interpretation of this finding is that both sources of error contribute to the deficit in ribosome numbers and that, in thicker sections, the increased number of superimpositions is compensated by the decrease in the proportion of ribosomes incompletely included in the section. It is likely that these artifacts do not distort very much the distribution pattern of ribosomes in the density equilibration experiment (Fig. 7). Indeed, in 40 nm thick sections, the polysomal grouping of ribosomes makes the likelihood of superimposition independent of the density of coating of the vesicles. Accordingly, the fit between the distribution of RNA and that of ribosomes in the gradient experiment (Fig. 7) is to be considered significant. In particular, it argues against the existence of sizable amounts of membrane RNA bound to smooth membranes.

Distribution of Ribosomes on Microsomal Membranes

As shown previously (1) and in this paper (Fig. 1), after density equilibration of microsomal elements in sucrose gradients, the distribution of RNA, although characterized by a distinctly higher median density, nevertheless overlaps considerably the distribution patterns of proteins and phospholipids. Two alternative hypotheses have been put forward by Amar-Costesec et al. (1) to account for this finding. Either a clear-cut distinction exists between rough-surfaced and smooth-surfaced vesicles and the two populations are characterized by broad and widely overlapping density frequency distributions, so that they cannot be clearly separated from each other on the basis of density alone; or all intermediates exist between pure smooth vesicles and vesicles fully coated with ribosomes, and the number of ribosomes per unit weight of membrane material plays a major role in determining the equilibrium density of a given vesicle.

The quantitative morphological analysis presented in this paper definitely rules out the first possibility and establishes firmly the existence of a continuous spectrum of microsomal vesicles, ranging from those that are entirely ribosome-free to those that are maximally coated with ribosomes. As regards the influence of the ribosome load on the equilibrium density of the rough vesicles, a detailed calculation is not possible, since the physical characters (osmotic behavior) of the particles have not been determined. However, knowing that a rough vesicle may be coated by several tens of ribosomes, it is obvious that the increase in equilibrium density with the ribosome load can be largely accounted for by the high density of ribosomes.

In the light of these results, and as already pointed out by de Duve (13), the significance of the preparations generally designated as "rough" and "smooth" microsomes must be cautiously evaluated. The various methods applied for the isolation of rough and smooth fractions must be considered as effecting a somewhat arbitrary cut in a continuous spectrum, at a point that may vary from one method to another.

Transitional elements between typical rough and smooth endoplasmic reticulum were repeatedly described. It is therefore not surprising to find microsomal vesicles that bear a small number of ribosomes. Furthermore, the variability of the ribosome load partly originates in the polysomic grouping of the ribosomes on the rough membranes. Owing to this discontinuous distribution, the breaking up of the rough membranes results in vesicles coated with a variable number of ribosomes and possibly supplies a number of smooth vesicles. Hence, even if it were feasible to isolate quite pure preparations of smooth vesicles, derived solely from the ER membranes, some of them could arise from the rough variety.

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REFERENCES

1. Amar-Costesec, A., H. Beaufay, E. Feytmans, D. Thines-Sempoux, and J. Berthet. 1969. Subfractionation of rat liver microsomes. *In* Microsomes and Drug Oxidations. J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts, and G. J. Mannering, editors. Academic Press Inc., New York, 41.
2. Bar, R. S., D. W. Deamer, and D. G. Cornwell. 1966. Surface area of human erythrocyte lipids: reinvestigation of experiments on plasma membrane. *Science* (*Washington*). **153**:1010.
3. Baudhuin, P. 1968. L'analyse morphologique quantitative de fractions subcellulaires. Thèse. Université de Louvain, Louvain, Belgium.
4. Baudhuin, P., and J. Berthet. 1967. Electron microscopic examination of subcellular fractions. II. Quantitative analysis of the mitochondrial population isolated from rat liver. *J. Cell Biol.* **35**:631.
5. Baudhuin, P., P. Evrard, and J. Berthet. 1967. Electron microscopic examination of subcellular fractions. I. The preparation of representative samples from suspensions of particles. *J. Cell Biol.* **32**:181.
6. Beaufay, H. 1966. La centrifugation en gradient de densité. Thèse. Université de Louvain, Louvain, Belgium, and Rockefeller University, New York.
7. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Canad. J. Biochem. Physiol.* **37**:911.
8. Blobel, G., and V. R. Potter. 1967. Studies on free and membrane-bound ribosomes in rat liver. I. Distribution as related to total cellular RNA. *J. Mol. Biol.* **26**:279.
9. Brux, B., and G. Richter. 1968. Ein Betrag zur Frage des Vorkommens einer RNS in den Membranen des endoplasmatischen Retikulums. *Acta Biol. Med. Ger.* **20**:527.
10. Dallner, G., A. Bergstrand, and R. Nilsson. 1968. Heterogeneity of rough-surfaced liver microsomal membranes of adult, phenobarbital-treated, and newborn rats. *J. Cell Biol.* **38**:257.
11. Dallner, G., and L. Ernster. 1968. Subfractionation and composition of microsomal membranes: a review. *J. Histochem. Cytochem.* **16**: 611.
12. Decloitre, F., and J. Chauveau. 1969. Isolation of two classes of rough vesicles from rat liver microsomes. *Fed. Eur. Biochem. Soc. Letters.* **2**:227.
13. de Duve, C. 1969. *In* Microsomes and Drug oxidations. J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts, and G. J. Mannering, editors. Academic Press Inc., New York. 57.
14. de Duve, C., B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans. 1955. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat liver tissue. *Biochem. J.* **60**:604.
15. El-Aaser, A. A., E. Reid, E. Klucis, P. Alexander, J. T. Lett, and J. Smith. 1966. Resolution of the components in the microsomal

fraction of liver in the B-IV zonal centrifuge. *Nat. Cancer Inst. Monogr.* **21**:323.

16. GARDNER, J. A. A., and M. B. HOAGLAND. 1968. A unique ribonucleic acid of low molecular weight from rat liver microsomes. *J. Biol. Chem.* **243**:10.
17. GILLIS, J. M., and WIBO, M. 1971. Accurate measurement of the thickness of ultrathin sections. *J. Cell Biol.* **49**:947.
18. HAMILTON, M. G., L. F. CAVALIERI, and M. L. PETERMANN. 1962. Some physicochemical properties of ribonucleoprotein from rat liver microsomes. *J. Biol. Chem.* **237**:1155.
19. HAMILTON, M. G., and M. E. RUTH. 1969. The dissociation of rat liver ribosomes by ethylenediaminetetraacetic acid; molecular weights, chemical composition, and buoyant densities of the subunits. *Biochemistry.* **8**:851.
20. HELMINEN, H. J., and J. L. E. ERICSSON. 1968. Studies on mammary gland involution. I. On the ultrastructure of the lactating mammary gland. *J. Ultrastruct. Res.* **25**:193.
21. HENNIG, A. 1956. Diskussion der Fehler bei der Volumbestimmung mikroskopisch kleiner kugeliger Körper oder Hohlraüme aus den Schnitt projektionen. *Z. Wiss. Mikrosk.* **63**:67.
22. ITO, A. 1968. Action of ribonuclease T-1 on smooth-surfaced vesicles of liver microsomes: evidence for the presence of membrane ribonucleic acid. *J. Biochem. (Tokyo).* **64**:699.
23. KARRER, H. E. 1960. Electron microscopic study of the phagocytosis process in lung. *J. Biophys. Biochem. Cytol.* **7**:357.
24. KEEPING, E. S. 1962. Introduction to statistical inference. D. Van Nostrand Company Inc., Princeton, N. J.
25. KING, H. W. S., and W. FITSCHEN. 1968. Characterisation of RNA from the smooth endoplasmic reticulum of rat liver. *Biochim. Biophys. Acta.* **155**:32.
26. KLEINIG, H. 1970. Nuclear membranes from mammalian liver. II. Lipid composition. *J. Cell Biol.* **46**:396.
27. LEIGHTON, F., B. POOLE, H. BEAUFAY, P. BAUDHUIN, J. W. COFFEY, S. D. FOWLER, and C. DE DUVE. 1968. The large-scale separation of peroxisomes, mitochondria, and lysosomes from the livers of rats injected with Triton WR-1339. Improved isolation procedures, automated analysis, biochemical and morphological properties of fractions. *J. Cell Biol.* **37**:482.
28. LILLIBRIDGE, C. B. 1968. Electron microscopic measurements of the thickness of various membranes in oxyntic cells from frog stomachs. *J. Ultrastruct. Res.* **23**:243.
29. LOUD, A. V. 1968. A quantitative stereological description of the ultrastructure of normal rat liver parenchymal cells. *J. Cell Biol.* **37**:27.
30. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
31. MAUNSBACH, A. B. 1966. Observations on the ultrastructure and acid phosphatase activity of the cytoplasmic bodies in rat kidney proximal tubule cells. With a comment on their classification. *J. Ultrastruct. Res.* **16**:197.
32. MUNNS, R., and T. HALLINAN. 1968. Rapidly labelled ribosomal RNA from the agranular microsomal membranes of rat liver. *Arch. Biochem. Biophys.* **127**:419.
33. REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**:208.
34. RICHARDS, E. G. 1968. A simplified method for the determination of the nucleotide composition of polyribonucleotides by spectrophotometric analysis. *Eur. J. Biochem.* **4**:256.
35. SCHNEIDER, W. C. 1957. Determination of nucleic acids in tissues by pentose analysis. *In* Methods in Enzymology. S. P. Colowick and N. O. Kaplan, editors. Academic Press Inc., New York. **3**:680.
36. SJÖSTRAND, F. S. 1963. A comparison of plasma membrane, cytomembranes, and mitochondrial membrane elements with respect to ultrastructural features. *J. Ultrastruct. Res.* **9**:561.
37. SMITH, C. S., and L. GUTTMAN. 1953. Measurement of internal boundaries in three-dimensional structures by random sectioning. *J. Metals.* **5**:81.
38. STÄUBLI, W., R. HESS, and E. R. WEIBEL. 1969. Correlated morphometric and biochemical studies on the liver cell. II. Effects of phenobarbital on rat hepatocytes. *J. Cell Biol.* **42**:92.
39. STOECKENIUS, W., and D. M. ENGELMAN. 1969. Current models for the structure of biological membranes. *J. Cell Biol.* **42**:613.
40. TASHIRO, Y., and P. SIEKEVITZ. 1965. Ultracentrifugal studies on the dissociation of hepatic ribosomes. *J. Mol. Biol.* **11**:149.
41. TASHIRO, Y., and D. A. YPHANTIS. 1965. Molecular weights of hepatic ribosomes and their subunits. *J. Mol. Biol.* **11**:174.
42. TATA, J. R., and H. G. WILLIAMS-ASHMAN. 1967. Effects of growth hormone and triiodothyronine on amino acid incorporation by microsomal subfractions from rat liver. *Eur. J. Biochem.* **2**:366.
43. THINES-SEMPOUX, D., A. AMAR-COSTESEC, H. BEAUFAY, and J. BERTHET. 1969. The association of cholesterol, 5′-nucleotidase, and alkaline phosphodiesterase I with a distinct

group of microsomal particles. *J. Cell Biol.* **43**:189.

44. TOMKEIEFF, S. I. 1945. Linear intercepts, areas, and volumes. *Nature (London).* **155**:24.
45. TULEGENOVA, L. S., N. P. RODIONOVA, and V. S. SHAPOT. 1968. Chemical fractionation of microsomes and metabolic activity of membrane RNA from rat liver. *Biochim. Biophys. Acta.* **166**:265.
46. WEIBEL, E. R. 1969. Stereological principles for morphometry in electron microscopic cytology. *Int. Rev. Cytol.* **26**:235.
47. WEIBEL, E. R., W. STÄUBLI, H. R. GNÄGI, and F. A. HESS. 1969. Correlated morphometric and biochemical studies on the liver cell. I. morphometric model, stereologic methods, and normal morphometric data for rat liver. *J. Cell Biol.* **42**:68.
48. WIBO, M., D. THINES-SEMPOUX, and A. AMAR-COSTESEC. 1970. Action sélective de la digitonine sur certaines membranes des cellules hépatiques. *In* Microscopie electronique, 1970. Résumés des communications présentées au VIIème congrès international, Grenoble. P. Favard, editor. Société Française de microscopie électronique. Paris. France. **III**:21.
49. WILSON, S. H., and R. V. QUINCEY. 1969. Quantitative determination of low molecular weight ribonucleic acids in rat liver microsomes. *J. Biol. Chem.* **244**:1092.
50. YAMAMOTO, T. 1963. On the thickness of the unit membrane. *J. Cell Biol.* **17**:413.