INTEGRATED STEREOLOGICAL AND BIOCHEMICAL STUDIES ON HEPATOCYTIC MEMBRANES

IV. Heterogeneous Distribution of Marker Enzymes on Endoplasmic Reticulum Membranes in Fractions

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ABSTRACT

The purpose of the study was to consider quantitatively the relationships between the surface area of the endoplasmic reticulum (ER) and constituent marker enzyme activities, as they occur in fractions collected from rat liver homogenates. The ER surface area was estimated in five membrane-containing fractions by use of a combined cytochemical-stereological technique (5), while, at the same time, ER marker enzymes were assayed biochemically. Fraction/homogenate recoveries for the ER enzymes averaged 100%, total membrane surface area 98%, and ER surface area 96%. Relative specific activities, which compare the relative amounts of ER marker enzyme activities to the relative ER surface area in the membrane-containing fractions, indicate variable distributions for glucose-6-phosphatase and NADPH cytochrome c reductase, but not for esterase.

The endoplasmic reticulum (ER) of rat liver hepatocytes has been studied extensively by use of marker enzymes to identify the presence of these membranes in fractions (17, 14, 15, 3, 1, 23, 10, 11, 21). In recent years, the question as to whether a given amount of marker enzyme activity is associated with a similar amount of ER membrane has become one of increasing importance (15, 25, 13, 44, 32, 8). Several papers have suggested that (a) marker enzymes are heterogeneously distributed on the ER and that (b) "ER marker enzymes" may also be attached to membranes other than those of the ER (15, 46, 2, 18, 22, 29, 24, 14, 41, 39, 4, 38, 30). The existence of such marker enzyme heterogeneities would be expected to have important consequences when one is interpreting bio-chemical data extrapolated from one fraction to the entire liver, or when one is making corrections for contaminations, particularly if the fractionation procedure leads to a sorting of the heterogeneous ER membranes.

In an earlier study (9), the surface areas of the hepatic membranes were determined in each of several fractions collected by differential centrifugation. An average 96% fraction-homogenate recovery for the membrane surface areas, accompanied by an average 95% recovery for several membrane marker enzymes, suggested that, for the most part, both membrane surface area and enzyme activity were being similarly conserved during the fractionation procedure. The study showed the extent to which homogenization forms a pool

of unidentifiable "smooth membranes," one that includes contributions from several different membrane organelles. A further study (35) indicated that the microsomal fraction was heavily contaminated by membranes other than those of the ER.

The purpose of the present study is to estimate the amount of one specific membrane type, namely the ER, in each of the fractions and then to see how its surface area relates to ER marker enzymes measured in the same fractions.

MATERIALS AND METHODS

ER Defined

The ER in the intact tissue hepatocytes is identified morphologically according to its appearance and location (9). The rough endoplasmic reticulum (RER) membranes carry ribosomes, are arranged into cisternae, and envelop the nucleus. The smooth endoplasmic reticulum membranes are ribosome-free extensions of the RER, form an anastomosing reticular network, and are connected frequently to elements of the Golgi apparatus (GA). In the membrane-containing fractions, vesicles and cisternae displaying the glucose-6-phosphatase (G6Pase) reaction product are assumed to be hepatocytic ER (42). Similarly, it is assumed that the G6Pase activities measured biochemically in these same fractions represent a marker enzyme attached to the ER. The GA, however, may also contain G6Pase activity (38, 30), and the potential effect of this additional location for the marker enzyme is considered in view of the assumptions above.

Preparation and Stereological Analysis of Control Samples

Methods for preparing samples and collecting data from electron micrographs of intact tissue and tissue fractions were described in the companion study (9) and a recent review (6).

Preparation of Fractions for G6Pase Cytochemistry

The cytochemical procedure for demonstrating the presence of G6Pase-containing membranes was based on the method of Leskes et al. (34). The tissue fractions identified according to de Duve (16), already characterized in the companion paper (9), were diluted with 0.1 M Na-cacodylate buffer (pH 6.6, 240 mosM, 20°C): 1:80 for the extract (E), 1:10 for the nuclear fraction (N), 1:10 for the heavy mitochondrial fraction (M), 1:5 for the light mitochondrial fraction (L), and 1:25 for the microsomal fraction (P). From each of these diluted fractions, a 0.1-ml aliquot was added to 1.5 ml of a Wachstein-Meisel reaction medium (43) which contained 1 mM D-glucose-6-phosphate (dipotassium salt, Sigma Chemical Co., St. Louis, Mo.), 2 mM lead nitrate, and 0.05 M Na-cacodylate buffer at pH 6.6. The osmolality of the reaction medium (120 mosM) was increased to 240 mosM by the addition of sucrose; the modification was introduced to maintain the microsomes in a spheroidal form. After a 20-min incubation at room temperature (20°C), the reaction was stopped by the addition of 2 ml of a 1% osmium tetroxide solution buffered with 0.1 M Na-cacodylate (pH 6.6, 240 mosM; adjusted with sucrose). The suspensions were filtered at a pressure of 1.5 atmospheres to produce pellicles, which were

secured in holders, and returned to an osmium tetroxide solution (see above) at 4°C for a total fixation time of 2 h. The pellicles were then washed sequentially with 0.1 M Na-cacodylate and M veronal acetate (pH 6.8) buffers (two 15-min changes for each), and refrigerated overnight in Na-cacodylate buffer. The following day, the pellicles were stained en bloc with uranyl acetate and prepared for electron microscopy as previously described (9). Cytochemical controls were treated in the same way, except that fractions were incubated in a substrate-free medium.

Quantitation of ER Membranes Identified Cytochemically

The pellicles treated cytochemically for G6Pase were sampled according to procedures already described for total pellicle membranes (9). Stereological methods were used to estimate the relative surface area of membranes labeled with G6Pase reaction product. Electron micrographs of pellicles derived from fractions E, N, M, and L were analyzed at × 120,000 and the P at × 150,000, using a square, double-lattice test system (1:9) with 36 coarse points, 27 × 27 cm. By counting intersections found by the test lines and the G6Pase-labeled (I_{er}) and all other unlabeled (I_o) membranes, the relative surface area (S_S) of ER membranes in a given fraction f is obtained by:

$$S_S(\text{er},f) = I_{er}/(I_{er} + I_o). \tag{1}$$

In the companion study, the aggregate membrane surface area per gram of liver ($S_w(\text{m},f)$) was estimated for each fraction (Table V in reference 9). By combining the data of this table with the results of Eq. 1, the surface area of the cytochemically labeled ER membranes in each fraction was obtained by:

$$S_w(\text{er},f) = S_w(\text{m},f) \cdot S_S(\text{er},f). \tag{2}$$

The estimates were then used to calculate ER membrane recoveries by summing $S_w(\text{er},f)$ for all fractions (N + M + L + P) and comparing these total surface areas of G6Pase-labeled membranes to those of the homogenate (E + N); in turn, both cytochemically based estimates were compared to those coming from electron micrographs of intact tissue where the ER membranes can be readily identified morphologically. The procedure is analogous to the one outlined in the earlier paper (9).

The membrane profiles (sections through vesicles) contained variable amounts of the cytochemical reaction product. When only a portion of a profile was labeled, the remaining segment was also scored as positive. In using such a binary convention (yes or no) for identifying membrane profiles as ER, it is assumed that the G6Pase enzymes are distributed across the entire surface of the membrane vesicles.

The estimates for the relative surface areas of the ER membranes (Eq. 1) were not corrected for the section thickness effect (45, 9) because it appears to introduce only a minor error. Because the labeled membrane profiles occurred predominantly, but not exclusively, as small vesicles, and the unlabeled membranes as somewhat larger vesicles, it is likely that the values for $S_S(\text{er},f)$ are slightly overestimated. $S_S(\text{er},P)$ was overestimated by ~3% and $S_S(\text{er},M)$ by 7%; the other fractions lie somewhere in between. Such errors are not expected to have an important effect on the interpretation of the data.

Biochemical Methods

Details of the differential fractionation procedures are given elsewhere (9). The same preparations used to characterize the

fractions morphologically were also assayed for membrane marker enzymes biochemically. These included G6Pase (26), esterase (19), and NADPH cytochrome c reductase (27, 28). Protein was determined according to Lowry et al. (36). The results of enzyme assays are expressed in international units (micromoles of substrate utilized or of product formed per minute).

RESULTS

Cytochemical Identification of ER Membranes in Fractions

Fractions treated cytochemically for G6Pase are illustrated in Figs. 1–4. The lead reaction product occurs on the inner surface of vesicular and cisternal profiles and displays a variable deposition ranging from continuous to widely separated. No reaction product was detected in the cytochemical controls.

Surface Area of the ER in Fractions

By combining the cytochemical identification of the ER in the fractions with the stereological estimates for that membrane surface area, the ER surface area was estimated in 15 fractions coming from three animals (Table I). Individual data points from each animal are included to illustrate the range of the estimates among the animals for a given fraction which, in some cases, varied by as much as a factor of two. Such differences are thought to be the result of variations from one experiment to another in the washing and decanting steps of the fractionation procedure. The microsomal fraction contained ~67% of the total ER membranes, the remaining 33% being distributed more or less equally among the N, M, and L fractions.

ER Recoveries: Intact Tissue-Homogenate-Fractions

Table II compares estimates for the ER surface areas determined from three different preparations of the same livers: intact tissue (T), homogenate (H), and fractions (F). The average values for the ER surface recoveries were F/H = 96%, H/T = 74%, and F/T = 71%.

Biochemical Data Recoveries

Units of activity and recoveries for three ER marker enzymes are given in Table III. Marker enzymes for other hepatocytic membranes determined in these same preparations have already been reported (9). The fraction-homogenate recov-

eries averaged 95% for G6Pase, 104% for esterase, 100% for NADPH cytochrome c reductase, and 98% for protein.

Integration of Biochemical and Morphological Data

The biochemical recovery (F/H) for G6Pase activity averaged 95.4% (Table III), while the cytochemically identified surface area of the ER was recovered at 95.8% (Table II). Given similar recoveries for the marker enzymes and ER surface areas, both of which were related to a comparable gram of identical livers, the data were linked to one another by plotting their relative distributions among the fractions. Essentially, this represents a modification of the relative specific activity (RSA) plot of de Duve (18), in that it substitutes the relative distribution of the ER surfaces for the relative protein distributions. These results are illustrated in Figs. 5–7.

G6Pase Activity vs. ER Surface Area

Fig. 5 represents a modified RSA plot comparing relative enzyme activity to relative membrane area. The figure illustrates the extent of the membrane heterogeneity among the fractions, which was found to be significant at the 0.026 probability level by use of an analysis of variance (40); Bartlett's test (40) for G6Pase and NADPH cytochrome c reductase, but not esterase, indicated homogeneous variances within the fractions. Of particular note is the observation that the differential fractionation procedure alone appears quite capable of collecting selectively ER membranes that are biochemically different. ER membranes in the earlier N and M fractions have a higher average concentration of enzyme activity per unit membrane area than those of the later L and P fractions. The range of the average marker enzyme heterogeneities among the four fractions was 1.54–0.86, representing a difference of ~80%. The extent of the heterogeneity in a given fraction from one experiment to another was likewise variable, particularly in the N, M, and L fractions.

Esterase Activity vs. ER Surface Area

The RSA plot shown in Fig. 6 suggests that of the four marker enzymes considered, esterase most closely approximates the condition of biochemical homogeneity; the analysis of variance detected no significant differences in RSAs among the four fractions; the significance level was 0.29. The data
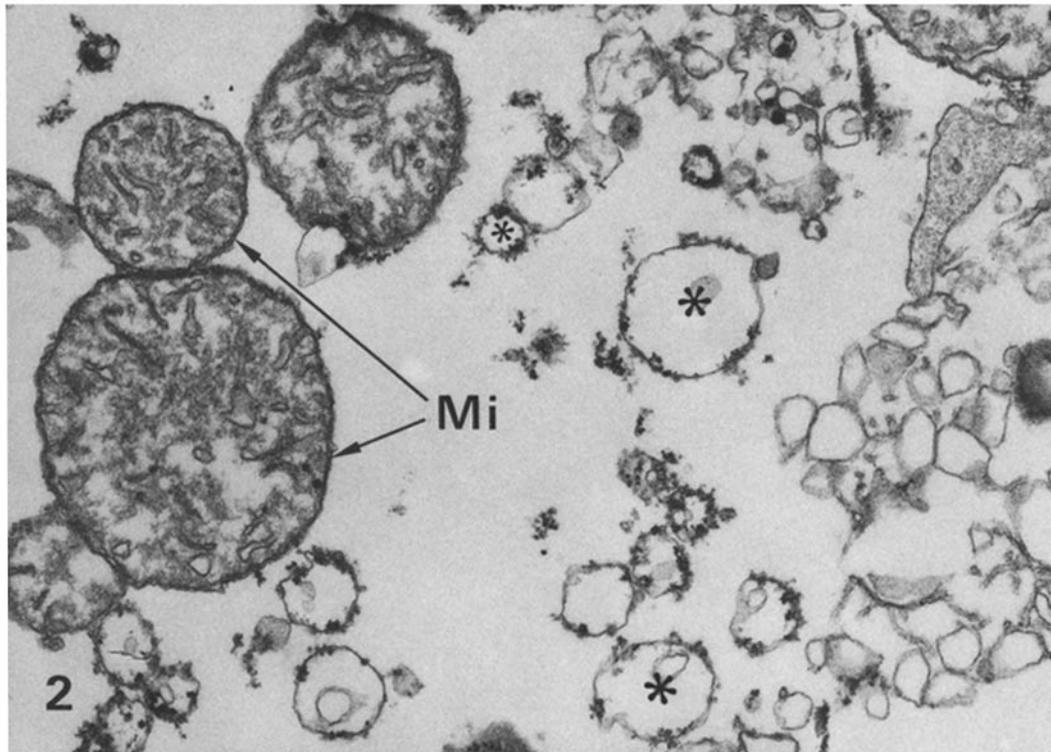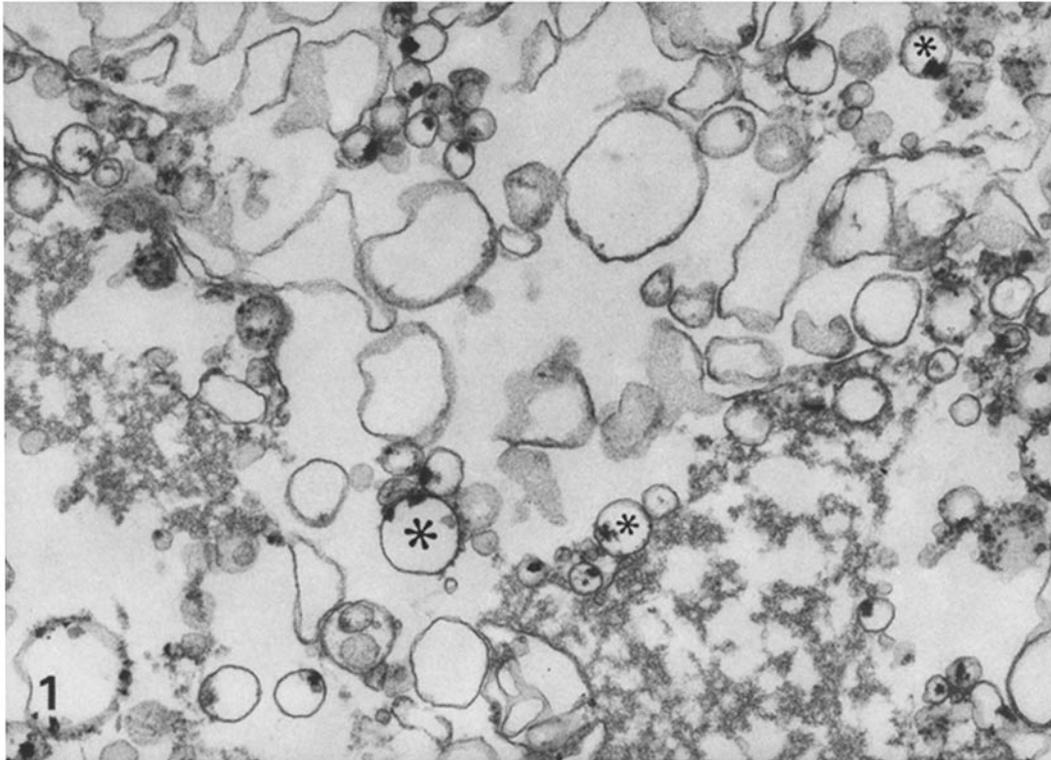
FIGURE 1 N fraction treated cytochemically for G6Pase. Examples of membrane vesicles originating from the ER (∗) contain the lead reaction product, whereas membranes derived from other sources do not. × 48,000.

FIGURE 2 M fraction treated for G6Pase. Again, examples of the ER vesicles containing the reaction product are indicated with an asterisk. Note the absence of the reaction product in the mitochondria (*Mi*). × 48,000.
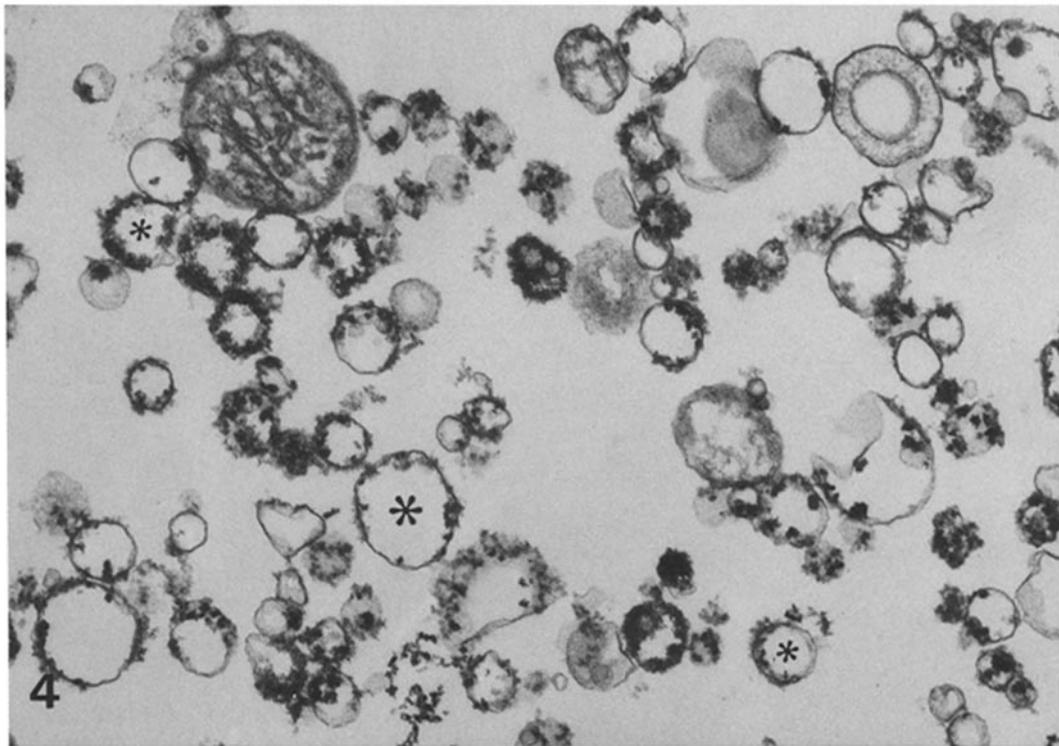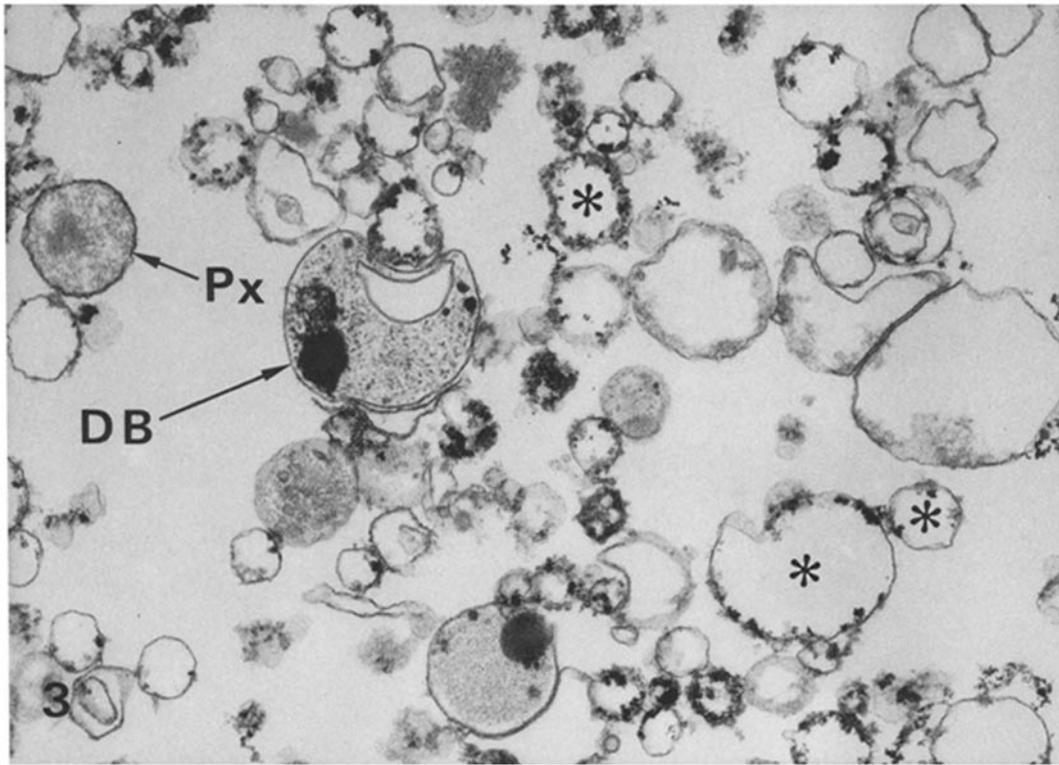
FIGURE 3   L fraction treated for G6Pase. The reaction product can be found within some of the membrane vesicles with varying degrees of intensity; examples are indicated (∗). Dense bodies (*DB*) and peroxisomes (*Px*) are identified. × 48,000.

FIGURE 4   P fraction treated for G6Pase. Many of the membrane vesicles contain the reaction product, reflecting the large proportion of ER. × 48,000.

Compartmental surfaces	Animal	E	N	M	L	P
Total membranes*	2‡	6.03	1.19	2.08	0.677	2.93
$S_{W(f)}$	3‡	6.72	1.56	1.82	0.401	4.16
	4	6.84	1.66	3.37	0.317	3.39
	$\bar{X} =$	6.53	1.47	2.42	0.465	3.49
	SE =	±0.252	±0.143	±0.479	±0.109	±0.359
Fraction of total membranes	2	0.496	0.240	0.202	0.682	0.580
G6Pase positive (in %)	3	0.455	0.307	0.115	0.562	0.627
$S_S(er, f)$	4	0.461	0.256	0.143	0.562	0.658
	$\bar{X} =$	0.471	0.268	0.153	0.602	0.622
	SE =	±0.0128	±0.020	±0.0256	±0.040	±0.0227
ER membranes*	2	2.99	0.287	0.421	0.462	1.70
$S_W(er, f)$	3	3.06	0.479	0.209	0.225	2.61
	4	3.15	0.427	0.480	0.178	2.22
	$\bar{X} =$	3.07	0.398	0.370	0.288	2.18
	SE =	±0.0463	±0.0573	±0.0823	±0.0879	±0.264

* Data in m^2/g liver corrected for 30-nm section thickness using factors given in Table II (Bolender et al., 1978, *J. Cell Biol.* 77:565).

‡ Data taken from animals 2 and 3 of earlier study (9).

Compartmental surfaces	Animal	T	H	F	Recoveries		
					F/H	H/T	F/T
		m^2/g			%		
Total membranes	2	9.49	7.23	6.88	95.2	76.2	72.5
	3	8.95	8.27	7.94	96.0	92.4	88.7
	4	9.22	8.51	8.74	102.7	92.2	94.7
	$\bar{X} =$	9.22	8.00	7.85	98.0	86.9	85.3
	SE =	±0.156	±0.393	±0.539	±2.38	±5.37	±6.63
ER membranes	2	4.53*	3.28	2.87	87.5	76.1	66.6
	3	4.32*	3.54	3.52	99.5	76.6	76.2
	4	4.46*	3.29	3.30	100.4	69.2	69.4
	$\bar{X} =$	4.44	3.37	3.23	95.8	74.06	70.7
	SE =	±0.0617	±0.0850	±0.191	±4.16	±2.39	±2.85

* ER membranes from nonhepatocytic cells not included.

from animals 3 and 4 suggest a higher esterase RSA in the L fraction compared with the N, but just the opposite was seen in animal 2.

NADPH Cytochrome c Reductase vs. ER Surface Area

The activity of NADPH cytochrome c reductase also appeared heterogeneously distributed across the membranes of the ER, as indicated by the RSA plot of Fig. 7. The N fraction, as was the case for the G6Pase (Fig. 5), showed the highest activity, whereas the L rather than the P, as seen in the earlier figure, contained the lowest. The analysis of variance suggested that the ER membranes in the four fractions were different, as indicated by a significance level of 0.003.

TABLE III

Biochemical Data from Fractions

Enzymes	E	N	M	L	P	S	Recovery
G6Pase	19.9	4.23	3.00	2.15	13.3	0.390	95.4
	±2.00	±0.590	±0.409	±0.240	±2.44	±0.045	±1.44
Esterase	180.0	27.0	25.0	20.2	115.0	24.5	104.0
	±28.8	±6.81	±3.82	±0.995	±17.5	±5.74	±5.96
NADPH cytochrome c re-	3.58	0.781	0.385	0.206	2.74	0.254	100.0
ductase	±1.29	±0.307	±0.125	±0.0236	±1.09	±0.0634	±1.11
Protein	189.0	55.2	52.9	9.52	38.6	83.2	98.0
	±4.51	±6.36	±5.55	±0.964	±3.93	±5.54	±0.670

Enzymes: U/g liver; proteins mg/g liver; $n = 3 \pm$ SE. 1 g of liver = 1.07 ml.
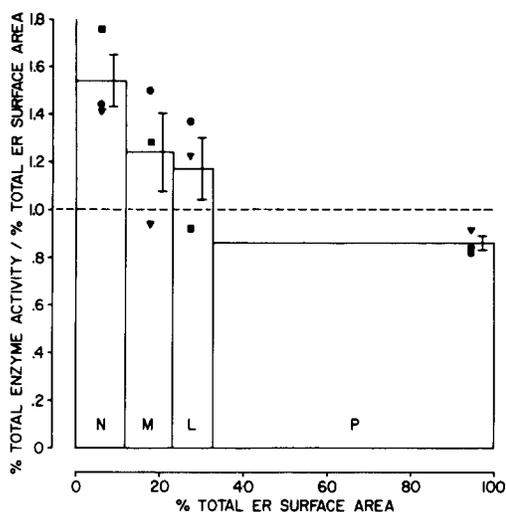
FIGURE 5 The RSA of G6Pase (% G6Pase/% ER surface) for the four membrane-containing fractions: nuclear (N), heavy mitochondrial (M), light mitochondrial (L), and microsomal (P). Means and standard errors are indicated and the dotted line at 1.0 is the prediction for biochemical homogeneity; animals: 2 (■), 3 (●), and 4 (▼).

DISCUSSION

The study has attempted to find quantitative relationships between the surface area of the ER and the activities of associated marker enzymes. Membrane-containing fractions prepared from liver homogenates were characterized according to ER marker enzymes and the surface areas of ER and total membranes. The interpretations of the integrated data were based on morphological and biochemical recoveries that compared the fractions to the original homogenate. With the cytochemically identified ER surface area as a reference, the results indicate that G6Pase and NADPH cytochrome c reductase are not uniformly distributed

across the membranes of the ER. The implication of this finding is that an ER marker enzyme activity may not, by itself, represent sufficient information (a) to determine the relative amount of ER in a given fraction or (b) to correct a fraction for an ER contamination.

Methods and Recoveries

The strategy of calculating recoveries for enzymes and membrane surface areas used earlier (9) was likewise applied to the ER membranes identified cytochemically. The data in Table II indicate that the combined stereological-cytochemical method gave a 95.8% F/H recovery for the surface area of the ER membranes, only 0.4% more than the recovery for G6Pase activity (Table III). Considerably lower values, however, were found for the tissue recoveries: H/T = 74%; F/T = 71%. These lower tissue recoveries are thought to result from an overestimate for the surface areas of the intact tissue membrane; the consequence of tissue shrinkage that occurs during the preparation for electron microscopy. A shrinkage correction factor has not yet been applied to the intact tissue data, but one accounting for approximately a 20% decrease in volume would elevate the tissue recoveries to the level of the F/H recoveries. Studies related to preparation artifacts indicate that a 20% specimen shrinkage is within the expected range (37, 20).

The tissue recoveries (H/T, F/T), however, even without the shrinkage correction provide a means for checking the reliability of the stereological-cytochemical method. The data in Table II indicate that the tissue recoveries for the total membranes averaged 86%, whereas the comparable recoveries for the ER membranes averaged only 72%. As the same intact tissue estimates were used for both recovery calculations, these two recoveries

were expected to be the same. Clearly, this was not the case, in that the recoveries differed by somewhat >10%. Although this difference cannot be explained, two possibilities can be considered: (a) It would appear that ~10% of the ER membranes are not being detected by the cytochemical identification procedure. Sections through ER vesicles might not include the lead reaction product required for the ER identification. Such losses would not seem to be of major importance in that the amount of membranes in the microsomal fraction identified as ER (62%) cytochemically compares favorably with an independent estimate for the ER (63%) based on freeze-fracture replicas (35). Beaufay and et al. (4) have reported a larger percentage of the ER in the P fraction, ~77%, but this higher value results from the fact that their fractionation procedure concentrates more of the ER in the microsomal fraction than the one used in this study. Their microsomal fractions, for example, contained 76% of the G6Pase activity of the membrane-containing fractions, whereas ours contained only 59%. (b) The membranes identified in the intact tissue electron micrographs as ER may not all be cytochemically positive for the G6Pase. The lead reaction product is not uniformly distributed adjacent to the ER membranes, and whether this reflects the distribution of the enzyme or is an artifact of the method remains an open question. Further clarification of this point may be possible by means of immunocytochemical techniques, particularly when they are combined with stereological methods (31). The current cytochemical interpretation, however, suggests that in the adult rat liver all the ER membranes are G6Pase positive (33).

ER Marker Enzyme Heterogeneities

It is generally accepted that the liver lobule consists of hepatocytes heterogeneous with respect to their structure (see for review, Reference 7) and function. It is therefore not surprising that membrane heterogeneities appear in fractions collected from such a nonhomogeneous source. The problem, of course, is to detect these heterogeneities quantitatively, and the purpose of this study has been to look for solutions by combining morphological and chemical techniques. The resulting data shown in Figs. 5–7 indicate that the ER membranes in the four fractions show differences with respect to the amount of marker enzyme activity per unit of ER surface area. The fact that slight variations in the preparation procedures are
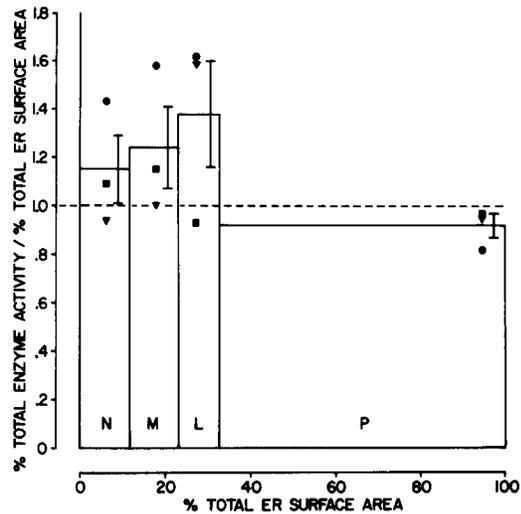
FIGURE 6 RSAs based on esterase activity and the surface area of the ER found in the four fractions are illustrated.
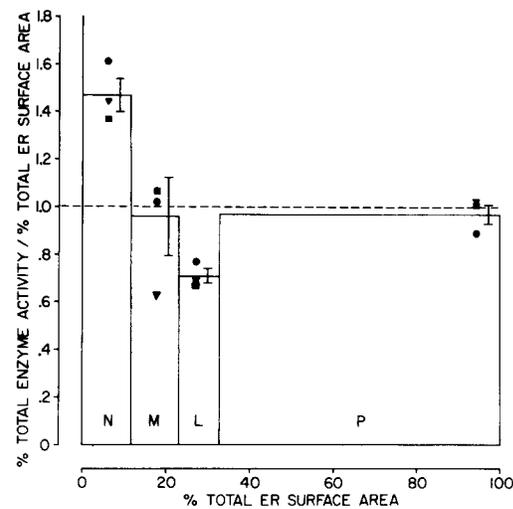
FIGURE 7 RSAs are illustrated which compare the activity of NADPH cytochrome c reductase to the surface area of the ER (see Fig. 5 for details).

capable of changing the extent of the heterogeneities, particularly in the earlier fractions, would seem to suggest that the heterogeneities reflect some physical property of the membranes in the fractions, a finding consistent with the views of both Beaufay et al. (4) and Dallner (13). But how can the ER membrane heterogeneities seen in Figs. 5–7 be explained? Presumably, they reflect differences in the structure and function of the ER within and/or between hepatocytes. Unfortu-

nately, neither the biochemical nor the stereological techniques of this study are able to resolve such differences. In fact, differences among the hepatocytes are very effectively "averaged" by both techniques. An integrated approach still seems to be the most likely way of obtaining an answer to this question, but both the biochemical and stereological techniques now need to be used at a level of resolution consistent with detecting both intra- and intercellular ER heterogeneities.

A practical application of the RSA data in Figs. 5–7 is that they can be used to estimate heterogeneity correction factors for the ER in the four fractions: a correction factor is defined as the reciprocal of the mean RSA value. A relative enzyme activity multiplied by the correction factor allows one to estimate the relative surface area of the ER in a given fraction. Table IV lists the correction factors for the three ER marker enzymes and illustrates the potentially large errors associated with an assumption of biochemical homogeneity when extrapolating data from a single fraction to the whole liver or when correcting a single fraction for contaminating ER.

Effect of Golgi G6Pase Activity

In interpreting the results of this study, it has been assumed that, for all practical purposes, G6Pase is a marker enzyme for the ER. A very small amount of this enzyme, however, has been found in association with membranes of the GA (38, 30). The surface area of the GA accounts for only ~2% of the combined ER-Golgi compartment (9), and its G6Pase contributes only ~0.2% to the total liver activity. (The RSA of the Golgi G6Pase is only 10% that of the ER [38], and its activity is quite labile [30]). Because these contributions of the GA and the ER are so small, they are not expected to have an important effect on our interpretations related to the ER. What seems to be far more important is the observation that G6Pase has now been found at another morpho-

logical location. Given the additional fact that Golgi membranes of the liver are continuous with those of the ER (12), the GA (or some portion thereof) might now be considered more correctly as a third type of ER. Its lower RSA for G6Pase, for example, could represent merely one of the several ER heterogeneities being uncovered in this and earlier studies.

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