STEREOLOGICAL ANALYSIS OF FREEZE-FRACTURED SUBFRACTIONS FROM SKELETAL MUSCLE

I. Relative Intrinsic Protein

II. Relative Lipid Content and Protein-to-Lipid Ratio

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ABSTRACT Standard microsomal subfractions from biological tissues are not homogeneous but mixtures of membranes derived from the various cellular organelles. In the case of skeletal muscle, freeze-fracture replicas show both smooth concave faces and concave faces densely populated with 90-Å particles. Stereological sampling techniques have been applied to such replicas and the relative surface area of sarcoplasmic reticulum (SR) membrane calculated. Expressions are derived that estimate the relative fraction of SR instrinsic protein and lipid as a function of the relative surface area. Although most of the protein in our subfractions is SR protein, a significant amount of lipid is non-SR lipid. The effect of this on measurements of the protein-to-lipid ratio is discussed.

INTRODUCTION

As an alternative to cytochemical determinations of the heterogeneity of cellular subfractions, morphometric techniques are being developed that sample membrane freeze-fracture faces to estimate the purity of such preparations (Weibel et al., 1976; Losa et al., 1978). This new analysis, called freeze-fracture stereology, enables one to estimate the surface area contributed by microsomes of a given cellular origin relative to the surface area contributed by all microsomes. Although this is a useful parameter, the present paper derives parameters from this that are more amenable to biochemical measurement: the relative intrinsic protein and the relative lipid. Subfractions of skeletal muscle consisting primarily of vesicles of sarcoplasmic reticulum (SR) are characterized and estimates of the relative intrinsic SR protein and lipid are given. Furthermore, the effects of contaminating membrane to measurements of the protein-to-lipid ratio are discussed.

STRUCTURAL MODEL

A microsome is a membranous artifact of subcellular debris resulting from the mechanical disruption of cells. They are generally spherical in shape and consist of a bilayer of lipids and associated proteins. The proteins are either incorporated into the lipid bilayer (intrinsic) or are peripheral (extrinsic) as surface proteins or solubilized within the lumen of the vesicles. It is generally believed now that the intrinsic proteins are visualized as 90-Å particles on freeze-fracture faces. In freeze-fracture stereology one counts particles inside a test area and

computes a particle density distribution for the preparation. We let "ρ" represent a measured density of 90-Å freeze-fracture particles. The relationship between a particle and a molecule of protein is probably not one-to-one in most cases and, as we have shown in SR microsomes, each particle represents an oligomer of several Ca^{2+}-ATPase polypeptides (Scales and Inesi, 1976).

The distribution of particles on the microsomal fracture-faces varies in accordance with the cellular origins of the membrane vesicles. Although this statement has not been proven rigorously, our experience with skeletal muscle indicates that two distinct classes of microsomes can be identified after sampling the concave fracture-faces and that these classes appear to derive from the SR and the transverse tubules (T) (see Discussion).

The lipids, being smaller than the 35-Å platinum replica grain, are not visualized separately. It is generally accepted that the lipid domains are represented by the uninterrupted platinum surfaces as evidenced by studies with model lipid systems (Deamer and Branton, 1970; Deamer et al., 1970).

Estimation of Relative Intrinsic Protein from Relative Surface Area

Consider a simple case where there is a mixture of only two membrane classes: α and β. Let a microsome of class α be characterized by an intrinsic protein density $\phi(\alpha)$, i.e., the amount of protein intercalated in the bilayer per unit membrane surface area (protein/μm^2).

If $S(\alpha)$ is the average surface area of α membranes in the subfraction, then the amount of α protein will be $\phi(\alpha)S(\alpha)$. The relative amount of α protein in a mixture containing α and β microsomes is $\Pi_\Pi(\alpha)$.

$$\Pi_\Pi(\alpha) = \frac{\phi(\alpha)S(\alpha)}{\phi(\alpha)S(\alpha) + \phi(\beta)S(\beta)}.$$

Dividing numerator and denominator by S (total) and noting that $S(\alpha)/S(\text{total}) \equiv S_S(\alpha)$, Weibel's parameter, the relative surface area of α,

$$\Pi_\Pi(\alpha) = \frac{\phi(\alpha)S_S(\alpha)}{\phi(\alpha)S_S(\alpha) + \phi(\beta)S_S(\beta)}.$$

Letting $\eta = [\phi(\beta)/\phi(\alpha)$ and since $S_S(\alpha) + S_S(\beta) = 1$,

$$\Pi_\Pi(\alpha) = \left[1 + \eta\frac{1 - S_S(\alpha)}{S_S(\alpha)}\right]^{-1}. \quad (1)$$

This expression is plotted in Fig. 1 for three values of η ($\eta = 0.4, 0.2, 0.1$).

Estimation of Relative Lipid Content from Relative Surface Area

Let $\delta(\alpha)$ and $\delta(\beta)$ be the surface density of lipid for class α and β respectively, i.e., the amount of lipid per unit membrane surface area (lipid/μm^2). The units can be arbitrary since these densities will only appear as a dimensionless ratio.

The relative amount of α lipid will be

$$\Lambda_\Lambda(\alpha) = \frac{\delta(\alpha)S(\alpha)}{\delta(\alpha)S(\alpha) + \delta(\beta)S(\beta)}.$$

To relate $\Lambda_A(\alpha)$ to Weibel's relative surface area $S_S(\alpha)$, we follow the same derivation described above for $\Pi_\Pi(\alpha)$. Therefore, defining the ratio $\delta(\beta)/\delta(\alpha) \equiv \nu$

$$\Lambda_A(\alpha) = \left[1 + \nu \frac{1 - S_S(\alpha)}{S_S(\alpha)}\right]^{-1}. \tag{2}$$

This is plotted in Fig. 2 for $\nu = 1.5, 2$.

METHODS

SR Preparations

Microsomes were prepared from selected white muscle of the rabbit hind leg by the method of Inesi (Eletr and Inesi, 1972).

Freeze-fracture

Samples for freeze-fracturing were prepared by washing 10 mg of SR protein in 80 mM KCL and 10 mM MOPS, pH 6.8, and resuspending in 1 ml of 60 mM KCL, 10 mM MOPS, and 20% glycerol. To reduce picture taking, the vesicles were packed by spinning again at 18,500 rpm for 1 h. Samples of the pellet were frozen in Freon-22 and stored in liquid nitrogen. A dilute preparation (i.e., without the extra centrifugation) was also frozen. Freeze-fracturing was done with a Balzers 360 M (Balzers Corp., Nashua, N.H.) at -100°C in a vacuum of 1×10^{-6} Torr.

All samples were examined in a Philips EM200 electron microscope at 80 kV.

Stereological Procedure

An implicit assumption of freeze-fracture stereology is that the microsomes vesiculate in a consistent fashion, i.e., they are always right side out. Others have reported significant (~10%) numbers of inside

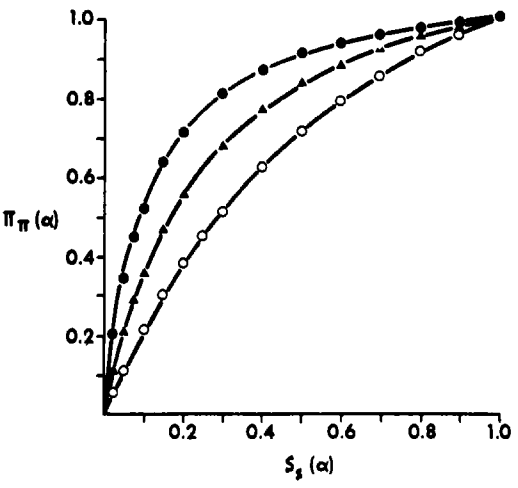

FIGURE 1

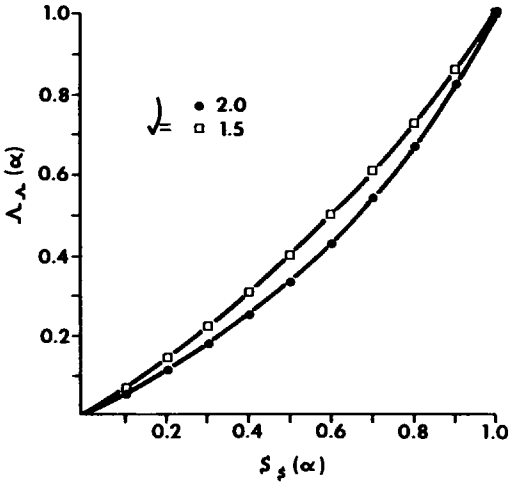

FIGURE 2

FIGURE 1 Calculated curves from Eq. 1 showing the dependence of the relative intrinsic protein $\Pi_\Pi(\alpha)$ on the relative surface area $S_S(\alpha)$ for three values of the dimensionless parameter η. (○) $\eta = 0.4$; (▲) $\eta = 0.2$; (●) $\eta = 0.1$.

FIGURE 2 Calculated curves from Eq. 2 showing the dependence of the relative lipid content $\Lambda_A(\alpha)$ on the relative surface area $S_S(\alpha)$ for two values of the dimensionless parameter ν. (●) $\nu = 2.0$; (□) $\nu = 1.5$.

out SR vesicles. However, no freeze-fracturing evidence was presented (Chevallier et al., 1977). In freeze-fracture, an inside out SR vesicle would display a high particle density on the convex fracture-face and the numbers of these would be a good estimate of the amount of inverted vesicles. Our preparations seldom demonstrate this kind of fracture-face (<1%). Inside out SR would display smooth concave fracture-faces, which in this kind of analysis, would be mistaken for T tubule vesicles.

As Weibel showed, sampling of only concave fracture faces with no cast shadow (~30% of all the concave faces) does not bias the results (Weibel et al., 1976). Sampling of these fracture-faces depends on prudent choices of the micrograph magnification M, the resolution or bin size of the particle density profile $\Delta\rho$, and the diameter of the test circle $2g$. For a difference of one particle in the test circle to appear in a different bin $\Delta\rho$, we choose the interval $\Delta\rho \simeq 1$ particle/test area $= 1/[\pi/4\ (2g)^2]$. The test circle diameter in millimeters is (3): $2g \simeq m\ \sqrt{4/(\pi\Delta\rho)}$, where $m = (M/1{,}000)$ and $\Delta\rho$ is expressed in particles/μm^2. M (or m) must be chosen to be high enough so that particles can be clearly distinguished and at the same time low enough so that many fracture-faces are accommodated in one micrograph (to reduce picture taking). $\Delta\rho$ must be chosen so that the test circle is smaller than most of the fracture-faces and yet not so small that particle sampling is inhibited. The choices for M and $\Delta\rho$ depend empirically on the characteristics of the particular subfraction being studied. In the case of muscle microsomes, M was chosen to be 175,000 and $\Delta\rho$ set at 500 particles/μm^2. Eq. 3 then gives $2g \simeq 8.8$ mm.

For each preparation 30–60 micrographs were recorded on Kodak 35-mm LR Film 2572 by systematically sampling the replicas at the corners of grid squares. This approach avoids sampling bias. The electron optical magnification was calibrated with a grating replica (882×10^{-3} μm; Ernest F. Fullam, Inc., Schenectady, N.Y.) and the print magnification was adjusted on the enlarger so that all micrographs were printed with the same final magnification.

9-mm test circles (corresponding to 0.05 μm) were drawn on the fracture-faces with a template (Berol Rapi Design No. 2140) and 150–200 samples were taken for each preparation. This gave a standard error for the particle density profile that was 2% of the mean particle density.

RESULTS AND DISCUSSION

The heterogeneity of SR preparations was dramatically revealed when dystrophic muscle was investigated (Sabbadini et al., 1975; Scales et al., 1977). Many concave fracture-faces had few or no particles in contrast to the high particle density expected for SR. After investigating the structure of the whole muscle we learned from thin sections that the T system was swollen compared to the normal animal and from freeze-fracture replicas that the T tubule fracture-faces contained few particles. This was first demonstrated in dystrophic muscle by Malouf and Sommer (1976). We then suspected that the heterogeneity of our microsomal preparation was due to this "extra" membrane. We devised a scheme to separate the low particle density microsomes from the SR in both normal and dystrophic preparations and identified these purified membranes by freeze-fracture stereological measurements and showed that they were, in fact, T tubular in origin (Sabbadini *et al.*, 1978; Scales and Sabbadini, 1979).

Fig. 3 shows a freeze-fracture replica of SR microsomes from rabbit muscle. Micrographs like this one were sampled with a test circle to generate the particle density distributions shown in Fig. 4. These three profiles were sampled from three separate microsomal preparations. The range of particle densities is less than that observed for microsomes obtained from the pectoralis major of the chicken where maximum densities of >10,000 particles/μm^2 were measured (Scales and Sabbadini, 1979).

Replicas of freeze-fractured whole muscle show that the cytoplasmic or P faces of the SR were populated with numerous 90-Å particles. Measured with the same test circle the

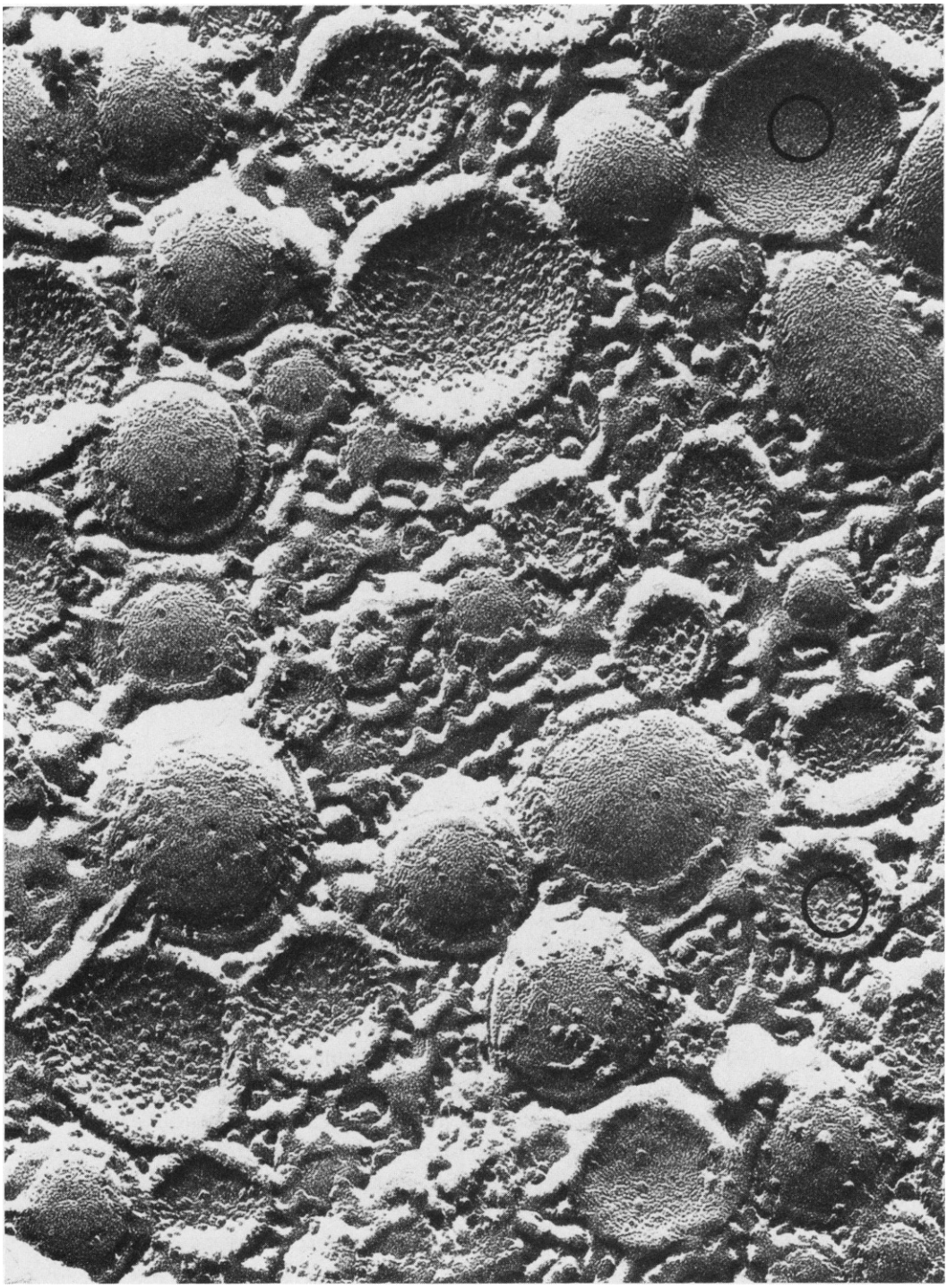

FIGURE 3 Freeze-fracture replica of microsomes from rabbit muscle. Particles are counted inside the 9-mm test circles and the concave fracture-faces (having no cast shadow) are categorized based on similar sampling of whole muscle replicas. Fracture-faces with two or fewer particles in the circle belong to the low particle density class (T tubule membrane, upper right) and fracture-faces with more than two belong to the high particle density class (SR, lower right). A convex fracture face with many particles can be seen at the bottom center; this may represent an inside out SR vesicle. × 175,000.

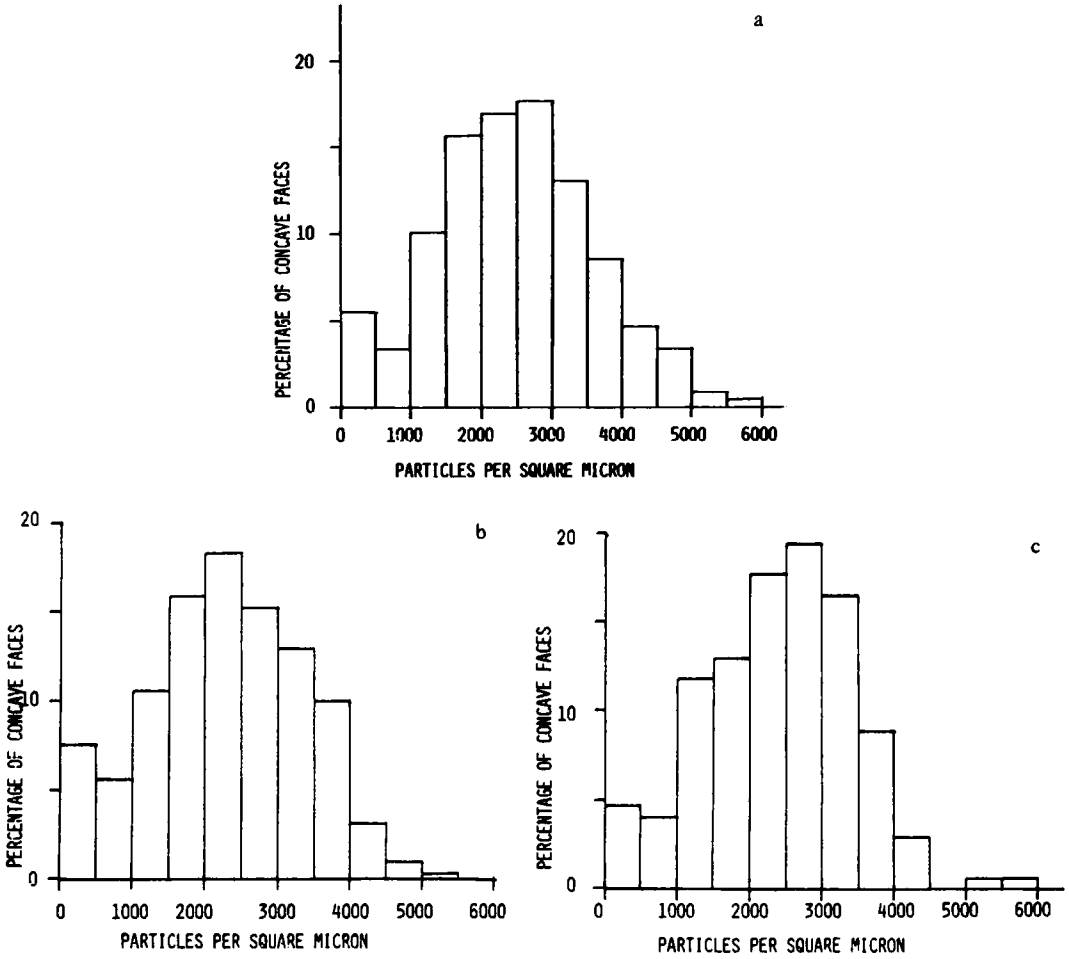

FIGURE 4 Particle density profiles measured with a test circle from shadow-free concave fracture-faces for three heterogenous SR subfractions. Two classes of fracture-faces are present: those with 1,000 particles/μm^2 or less and those with >1,000 particles/μm^2.

minimum number of particles for SR was three and the maximum 12, corresponding to a density range of 1,000–6,000/μm^2. Nowhere along the cytoplasmic fracture-faces of the SR network were there smooth regions. The microsomal preparations exhibit particle densities below 1,000/μm^2 and based on replicas of intact muscle, originate from non-SR membranes, most likely from T tubules. Assuming this to be the case, we divide the microsomal fracture-faces into two classes: low particle density, <1,000 particles/μm^2 and high particle density, >1,000 particles/μm^2. A similar distribution was obtained from a dilute preparation, i.e., one without the additional centrifugation for packing the microsomes as described in Methods. This indicated that the extra centrifugation did not select out any class of vesicle, which would, of course, bias the sampling. The mean densities of particles in the high density class were 2,820/μm^2 for preparation A, 2,710/μm^2 for B, and 2,740/μm^2 for C.

Relative Surface Area, S_S (SR)

Weibel shows that $S_S(\alpha)$ is equal to the relative number of shadow-free concave fracture-faces, $N_N(\alpha)$ multiplied by a size-dependent parameter, $\lambda(\alpha)/\lambda$ (total) (Weibel et al., 1976). λ is calculated from the size distribution of the concave fracture-faces $f(q)$, where $f(q)$ is the fraction of concave faces with diameter q ($= \sqrt{2}\, d/2g$):

$$\lambda = \frac{E(q^2)}{E(\sqrt{q^2 - 1}) - \dfrac{E(q)}{\sqrt{2}}},$$

where

$$E(q) = \Sigma f(q) \cdot q$$
$$E(q^2) = \Sigma f(q) \cdot q^2$$
$$E(\sqrt{q^2 - 1}) = \Sigma f(q) \cdot \sqrt{q^2 - 1}.$$

From these we calculate S_S (SR):

$$S_S(\mathrm{SR}) = N_N(\mathrm{SR}) \cdot \frac{\lambda(\mathrm{SR})}{\lambda(\mathrm{Total})}.$$

λ (total) is the size distribution parameter for all shadow-free concave faces.

The values for N_N(SR), λ(SR)/λ(total) and S_S(SR) for three microsomal preparations of the rabbit are shown in Table I. On the average, ~90% of the vesicles in our preparations appear to originate from the sarcoplasmic reticulum and 10% from non-SR membranes (T tubules). In cardiac preparations (to be reported later) only 80% of the vesicles appear to be SR and in preparations from dystrophic chicken (data not shown) only 54% of the vesicles originate from the SR. The relative number of SR vesicles in our skeletal muscle preparations varies slightly (5%) with the preparations. This is most likely due to the experimental errors associated with muscle selection and other subjective decisions made during the isolation. After the small correction for relative vesicle size, the relative surface area of SR vesicles in our preparation is also ~90%.

Relative Protein, Π_Π (SR)

Eq. 1 relates the relative surface area and the parameter η to the relative protein, where $\eta = \phi(\mathrm{T})/\phi(\mathrm{SR})$.

The actual value of η depends on the ratio of molecular weights of the intrinsic protein per

TABLE I
RELATIVE SURFACE AREAS FOR THREE SR PREPARATIONS

	N_N(SR)	λ(SR)/$\lambda_{(\mathrm{total})}$	S_S(SR)
SR (A)	0.911	0.993	0.905
SR (B)	0.869	0.999	0.868
SR (C)	0.912	0.999	0.911

unit membrane surface area. As a first approximation we assume that the amount of protein that corresponds to an intramembranous particle is the same for SR and T membranes. This estimate can be refined after the molecular weight of the T tubule protein is determined. Preliminary evidence obtained from purified normal and dystrophic chicken T tubule microsomes indicates that the major intrinsic protein is a Ca^{2+}-ATPase (Scales and Sabbadini, 1979). Assuming that this is also true for the rabbit, the ratio of particle densities is a reasonable estimate of η.

SR is an asymmetric membrane with few particles appearing on the E fracture-face in whole muscle. We therefore take the average particle density of the concave faces (with $\rho > 1{,}000/\mu m^2$) to represent ϕ(SR). The T tubule membrane, however, is symmetric, i.e., the P and E faces of whole muscle T tubules have nearly identical particle densities. We can estimate ϕ(T) by doubling the mean particle density of the low density concave faces ($\rho < 1{,}000/\mu m^2$). The values of η calculated this way are shown in Table II. The relative protein Π_Π(SR) calculated from S_S(SR) and η is also shown in Table II. The mean relative intrinsic protein for these preparations is 0.95. In other words, 95% of the intrinsic protein is SR protein and 5% is non-SR. Note that 90% of the surface area accounts for 95% of the intrinsic protein.

For relatively pure SR preparations ($S_S > 0.8$). Figure 1 shows that Π_Π is fairly insensitive to η. Doubling η changes Π_Π by 5% or less. This is some justification to the particle density approximation made in the estimation of η. The error in Π_Π(SR) due to errors in η is greater for impure preparations like those from the dystrophic chicken where S_S(SR) = 0.54 (data not shown).

Relative Lipid, Λ_Λ (SR)

Eq. 2 relates the relative surface area and the parameter ν to the relative lipid, where $\nu = \delta(T)/\delta(SR)$. Consider the following oversimplified model for SR and T microsomes. Assume that SR vesicles consist essentially of a monolayer of lipid, the external leaflet being mostly protein (Scales and Inesi, 1976). T vesicles consist essentially of a bilayer of lipid, both internal and external leaflets only sparsely populated with protein. Therefore, a reasonable (maximum) value for ν would be $\nu = 2$. Values of Λ_Λ(SR) are included in Table II, for the SR preparations.

Even for relatively pure SR preparations (say S_S [SR] > 0.9), a significant fraction of the total lipid is not derived from SR. In the examples shown, 17–24% of the lipid is in the form of T system vesicles. This becomes significant if one measures a lipid-related parameter in such heterogeneous preparations. Consider the protein-to-lipid ratio.

TABLE II
EXPECTED ERRORS IN PROTEIN-TO-LIPID RATIO FOR THREE SR PREPARATIONS

	S_S(SR)	η	Π_Π(SR)	Λ_Λ(SR)	$\frac{\Pi_\Pi(SR)}{\Lambda_\Lambda(SR)} - 1$
SR (A)	0.905	0.390	0.961	0.826	0.162
SR (B)	0.868	0.384	0.945	0.767	0.232
SR (C)	0.911	0.467	0.956	0.837	0.143

Protein-to-Lipid Ratio

The protein-to-lipid ratio for a mixture of microsomes from SR and T tubules is $R(\mathrm{SR, T})$, given by

$$R(\mathrm{SR, T}) = \frac{\phi(\mathrm{SR})S(\mathrm{SR}) + \phi(\mathrm{T})S(\mathrm{T})}{\delta(\mathrm{SR})S(\mathrm{SR}) + \delta(\mathrm{T})S(\mathrm{T})}.$$

Dividing by S(total),

$$R(\mathrm{SR, T}) = \frac{\phi(\mathrm{SR})S_S(\mathrm{SR}) + \phi(\mathrm{T})S_S(\mathrm{T})}{\delta(\mathrm{SR})S_S(\mathrm{SR}) + \delta(\mathrm{T})S_S(\mathrm{T})} = \frac{\phi(\mathrm{SR})}{\delta(\mathrm{SR})} \cdot \frac{\Lambda_A(\mathrm{SR})}{\Pi_\Pi(\mathrm{SR})}.$$

But $\phi(\mathrm{SR})/\delta(\mathrm{SR}) = R(\mathrm{SR})$, since $\Lambda_A(\mathrm{SR}) = \Pi_\Pi(\mathrm{SR}) = 1$ for pure SR microsomes. Therefore,

$$R(\mathrm{SR}) = R(\mathrm{SR, T}) \cdot \frac{\Pi_\Pi(\mathrm{SR})}{\Lambda_A(\mathrm{SR})}. \tag{3}$$

In other words, the protein-to-lipid ratio measured biochemically in an unpurified preparation needs to be corrected by the factor $\Pi_\Pi(\mathrm{SR})/\Lambda_A(\mathrm{SR})$. For relatively pure preparations ($S_S >$ 0.9) like the ones described here, the correction is significant. The last column in Table II shows the calculated values of $(\Pi_\Pi/\Lambda_A) - 1$ for the three SR preparations. This gives the fractional error of a measurement of the protein-to-lipid ratio of SR made from each preparation. For preparation A a determination of the protein-to-lipid ratio for SR would be underestimated by 16% because >17% of the total lipid present is non-SR.

Conceding the approximation of our calculations, the results elucidate the magnitude of the errors possible in certain biochemical assays of heterogeneous subfractions.

I gratefully acknowledge the assistance of Susan Keating for preparing the SR subfractions and for other technical assistance. Freeze-fracturing was made possible by the cooperation of the Electron Microscope Laboratory of the University of California at Berkeley. I thank the Research Committee of the University of the Pacific School of Dentistry for providing funds to attend a stereology course at the Marine Biological Laboratory in Woods Hole, Mass.

This work was supported by grants 5-PO1-HL-16607 and 5-SO7-RR05301 from the U.S. Public Health Service.

Received for publication 22 April 1980 and in revised form 4 October 1980.

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